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Involvement of Ca^{2+} in Regulation of Physiological Indices and Heat Shock Factor Expression in Four *Iris germanica* Cultivars under High-temperature Stress

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Abstract. **Although tolerance to high temperature is crucial to the summer survival of *Iris germanica* cultivars in subtropical areas, few physiological studies have been conducted on this topic previously. To remedy this, this study explored the physiological response and expression of heat shock factor in four *I. germanica* cultivars with varying levels of thermotolerance. The plants' respective degrees of high-temperature tolerance were evaluated by measuring the ratio and area of withered leaves under stress. Several physiological responses to high temperatures were investigated, including effects on chlorophyll, antioxidant enzymes, proline, and soluble protein content in the leaves of four cultivars. $CaCl_2$ was sprayed on 'Gold Boy' and 'Royal Crusades' considered being sensitive to high temperatures to study if Ca^{2+} could improve the tolerance, and $LaCl_3$ was sprayed on 'Music Box' and 'Galamadrid' with better high-temperature tolerance to test if calcium ion blocker could decrease their tolerance. Heat shock factor genes were partially cloned according to the conserved region sequence, and expression changes to high-temperature stress with $CaCl_2$ or $LaCl_3$ treatments were thoroughly analyzed. Results showed that high temperature is the primary reason for large areas of leaf withering. The ratio and area of withered leaves on 'Music Box' and 'Galamadrid' were smaller than 'Gold Boy' and 'Royal Crusades'. $CaCl_2$ slowed the degradation of chlorophyll content and increased proline and soluble protein in 'Gold Boy' and 'Royal Crusades' but had no significant effect on activating peroxidase or superoxide to improve high-temperature tolerance. Genetic expression of heat shock factor in 'Gold Boy' and 'Royal Crusades' was upregulated by Ca^{2+} at later stages of leaf damage under high-temperature stress. $LaCl_3$ down-regulated the physiological parameters and expression level of heat shock factor in 'Music Box' and 'Galamadrid'. These results suggest that different *I. germanica* cultivars have varying high-temperature tolerance and furthermore that Ca^{2+} regulates their physiological indicators and expression level of heat shock factor under stress.**

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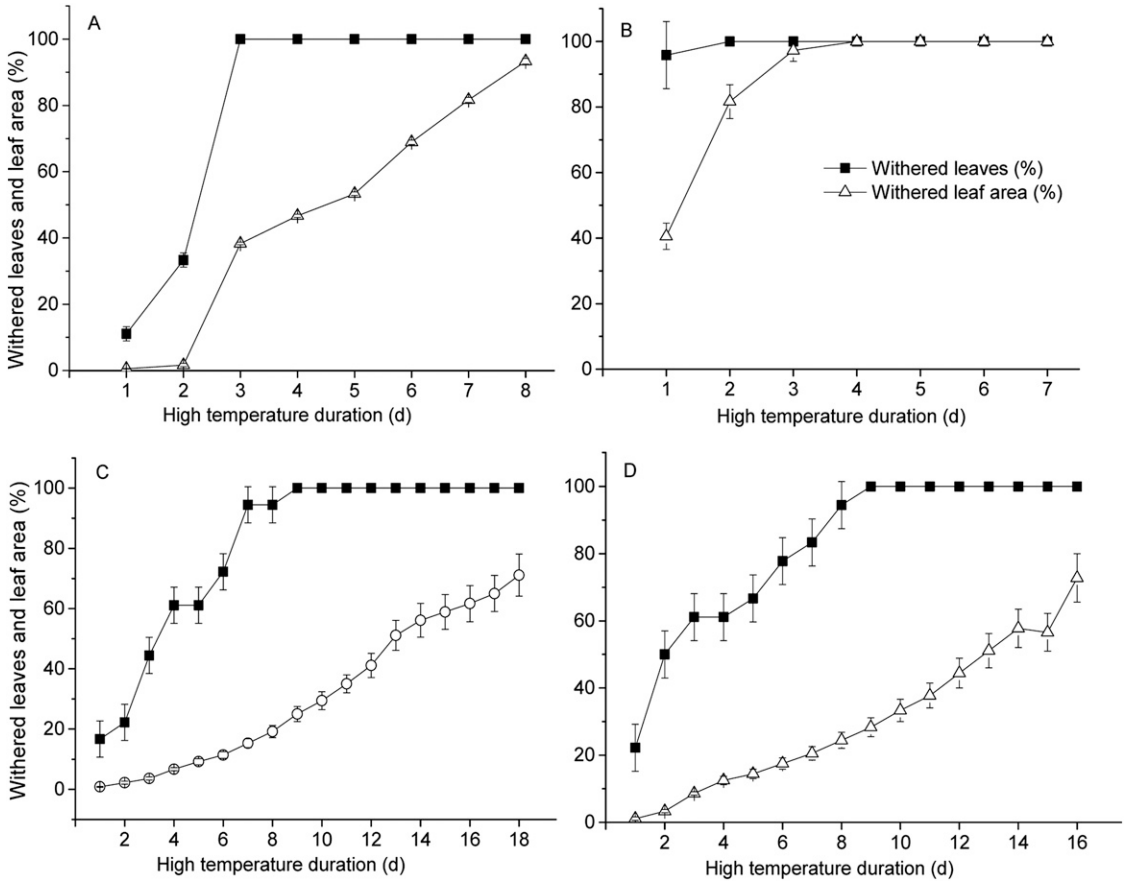

Fig. 1. Percentage of withered leaves and ratio of withered leaf area. Potted plants of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid] were treated with high temperatures at 40/30 °C (day/night) stress. Data of percentage of withered leaves were recorded as the percentage of withered leaves in total leaf numbers for each plant. The ratio of withered leaf area included the withered area divided by the total area of each leaf. Values represent the mean ± SE (n = 3) per treatment for each cultivar, respectively. The x-axis shows the duration of high-temperature duration by days.

As global warming intensifies, the high-temperature stress response of plants has become a key research topic worldwide (Wahid et al., 2007). High-temperature stress often causes a series of morphological, physiochemical, and genetic changes in plants, which may decrease their ornamental and economic value. Inside the plants, reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydroxyl radicals can be affected by stress (Schwanz and Polle, 2001). Although ROS damage essential cellular components such as DNA, protein, and lipids, plants have developed complex defense mechanisms against these oxidative stressors in the form of antioxidant enzymes such as peroxidase (POD) and superoxide (SOD) (Martindale and Holbrook, 2002). Altered activities of these antioxidant enzymes have been commonly reported in plants and are frequently used as indicators of high-temperature tolerance (Shi et al., 2006; Wahid et al., 2007).

Among the many physiological changes that occur in plants, genetic manipulation is regarded as the most critical process affected by high temperatures. When plants suffer high-temperature stress, they display a rapid increase in the level at which they express heat shock genes and a rapid accumulation of heat shock proteins. The expression of heat shock proteins is regulated by heat shock transcription factor (HSF) (Nover et al., 2001; Sangster and Queitsch, 2005). The conservation of heat shock transcription factors is the main regulatory measure of heat stress-responsive genes, which encode molecular chaperones (Wu, 1995). Studies show that HSF is involved in the induction of heat shock gene transcription in many plants such as tomato (*Solanum lycopersicum*), arabidopsis (*Arabidopsis thaliana*), and lily (*Lilium longiflorum*) (Mishra et al., 2002; Sarge et al., 1993; Yi et al., 2012).

HSFs are the terminal components of the heat shock signal transduction chain, whereas calcium has been identified in the upstream of HSF (Mittler et al., 2012). Calcium, a universal secondary messenger in plants, has been found to affect the regulation of physiological and biochemical processes in response to high-temperature stress (Hashimoto and Kudal, 2011; Wang et al., 2009). Calcium ions are known to contribute to structural and functional maintenance of plant cell membranes under abiotic stress. Application of $CaCl_2$ has been found to improve high-temperature tolerance in several plants by increasing antioxidant enzyme activity and reducing the lipid peroxidation of cell membranes (Bhattacharjee, 2008; Tan et al., 2011; Wang et al., 2009). Furthermore, specific calcium ion channel blockers such as $LaCl_3$ typically indicate the function of Ca^{2+} in modulating cell metabolism. $LaCl_3$ may decrease

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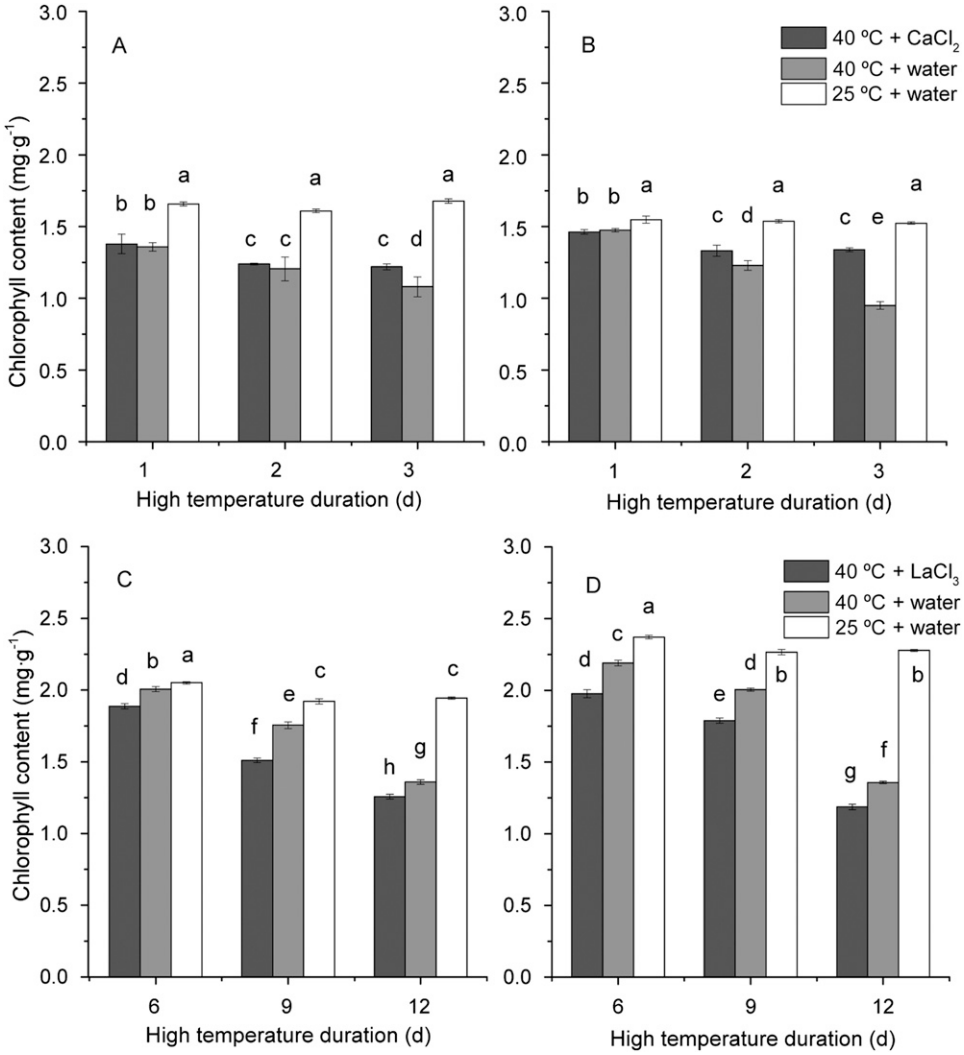

Fig. 2. Chlorophyll content in the leaves of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Potted plants were treated under a high-temperature stress of 40/30 °C (day/night) and sprayed with 100 mL distilled water (40 °C + water), 20 mM $CaCl_2$ (40 °C + $CaCl_2$), or 20 mM $LaCl_3$ (40 °C + $LaCl_3$) everyday. Plants incubated in 25/20 °C (day/night) and supplied with 100 mL distilled water everyday served as the control (25 °C + water). 'Gold Boy' and 'Royal Crusades' were sampled at 1, 2, and 3 d. 'Music Box' and 'Galamadrid' were sampled at 6, 9, and 12 d. The x-axis shows the duration of high-temperature duration by days. Values represent the mean ± SE (n = 3) per treatment. Different letters indicate significant differences at $P < 0.05$ levels.

thermotolerance by inhibiting the influx of extracellular Ca^{2+} in plants (Gong et al., 1997; Graziana et al., 1988).

Iris germanica is an ornamental plant known for its attractive variations in color and shape. It has been increasingly cultivated in gardens and public landscapes with warmer temperatures. In southern China, high summer temperatures are considered the most severe impediment to *I. germanica* production. Daytime temperatures frequently exceed 40 °C, withering leaves and reducing plant survival rates. However, the morphological, physiochemical, and molecular responses to high temperature in *I. germanica* have not been thoroughly investigated.

Our institute has collected more than 20 *I. germanica* cultivars from different parts of the world since 2009. One of our primary goals is the selection and cultivation of adaptive varieties for the subtropical area of the Chinese ornamental plants market. Before this present study, we previously tested on high-temperature tolerance of 16 different *I. germanica* cultivars. The results revealed that different cultivars show varying high-temperature tolerance with different speeds of leaf withering under high-temperature stress. 'Gold Boy' and 'Royal Crusades' were the two cultivars found with high sensitivity to high-temperature stress, whereas 'Music Box' and 'Galamadrid' showed strong tolerance to high temperatures.

The aim of this study is to further explore and confirm these differences in high-temperature tolerance and examine specifically whether the calcium ion is involved in coordination with physiological indices including antioxidant contents, chlorophyll, protein content, free proline and malondialdehyde (MDA), and/or the HSF transcriptional expression during high-temperature stress. Addressing the physiological and molecular mechanisms involved in heat tolerance will help researchers develop *I. germanica* cultivars that can better adapt to climate change.

Materials and Methods

Plant materials. One-year-old plants of four cultivars, including Gold Boy, Royal Crusades, Music Box, and Galamadrid, were selected and grown in pots sized 120 × 110 cm (diameter and height) and incubated in a climate chamber with a 16-h (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/8-h light/dark photoperiod, 80% relative humidity, and 25/20 °C (day/night) thermoperiod (control temperature). Individual plants per pot were prepared for further experiment. A commercial media of high-quality peatmoss (Fafard Growing Mix Canadian Sphagnum Peat Moss®; Sun Gro Horticulture, Agawam, MA) was used. Healthy plants with uniform size (three to five leaves each and rhizome lengths of 5.0 to 8.0 cm) were prepared for experiments.

Stress treatments. To compare the speed of leaf withering among different cultivars, high-temperature stress treatment was executed. High temperature was applied to plants at 40/30 °C (day/night), which were given 100 mL distilled water daily. As a control, the plants sprayed with 100 mL water were prepared and kept in a climate chamber at 25/20 °C (day/night). Each treatment was repeated three times by using three plants per replicate for every cultivar. The withering leaf was noted as data when the tip exhibited a yellow and brown color. During incubation, the leaf withering speed was evaluated in terms of percentage of withered leaves and ratio of the withered leaf area. The percentage of withered leaves was calculated based

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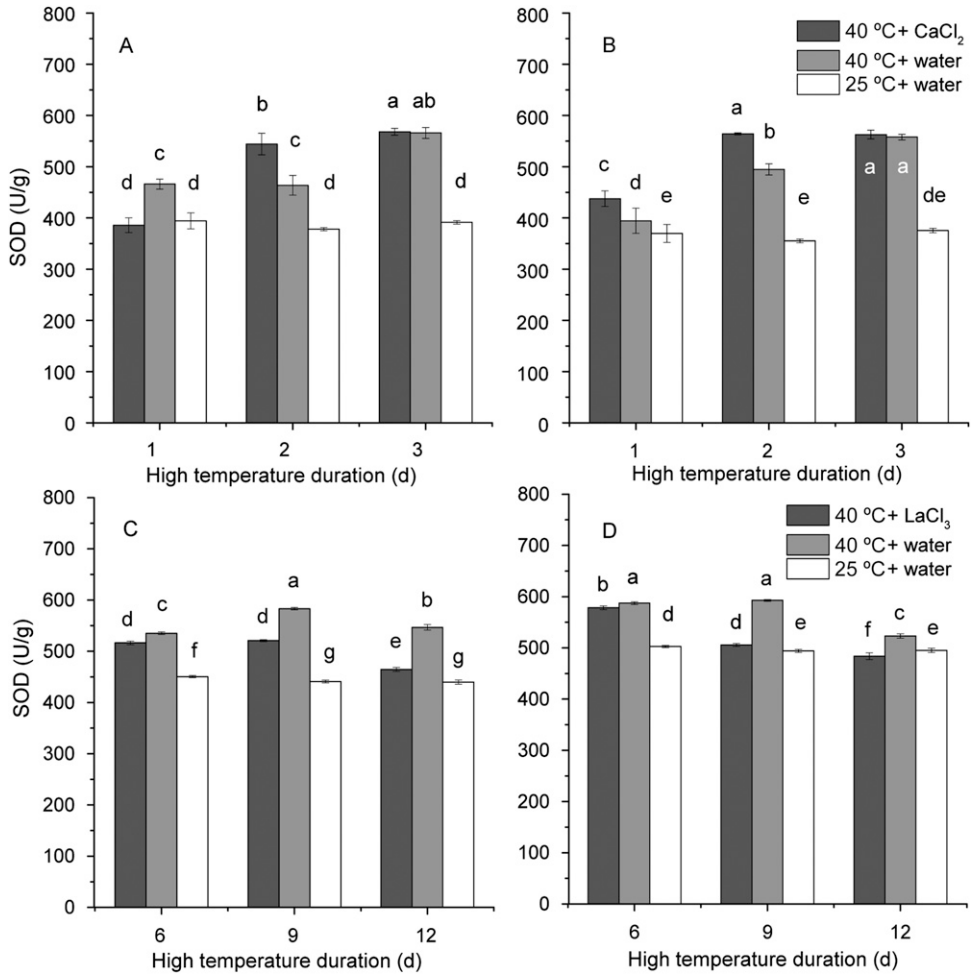

Fig. 3. Superoxide (SOD) content in the leaves of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Potted plants were treated under a high-temperature stress of 40/30 °C (day/night) and sprayed with 100 mL distilled water (40 °C + water), 20 mM $CaCl_2$ (40 °C + $CaCl_2$), or 20 mM $LaCl_3$ (40 °C + $LaCl_3$) everyday. Plants incubated in 25/20 °C (day/night) and supplied with 100 mL distilled water everyday served as the control (25 °C + water). 'Gold Boy' and 'Royal Crusades' were sampled at 1, 2, and 3 d. 'Music Box' and 'Galamadrid' were sampled at 6, 9, and 12 d. The x-axis shows the duration of high-temperature duration by days. Values represent the mean ± SE (n = 3) per treatment. Different letters indicate significant differences at $P < 0.05$ levels.

on the number of withered leaves as a percentage of the total number of leaves on each plant. The ratio of the withered leaf area was based on the withered area of the leaf divided by the total area of each leaf.

For the analysis of physiological index including antioxidant contents, chlorophyll, protein content, free proline, and MDA, the plants sprayed with 100 mL distilled water (20 mM $CaCl_2$ or 20 mM $LaCl_3$, respectively) were exposed to high-temperature stress at 40/30 °C (day/night). There were three replicates per treatment and three plants per replicate for each cultivar.

Leaves that remained green and alive were sampled for physiological index and HSF expression analysis at 1, 2, and 3 d for 'Gold Boy' and 'Royal Crusades' and 6, 9, and 12 d for 'Music Box' and 'Galamadrid'.

Physiological indices analysis. To investigate whether calcium is involved in the high-temperature tolerance of *I. germanica*, $CaCl_2$ was sprayed on 'Gold Boy' and 'Royal Crusades'. These two cultivars are considered to be particularly sensitive to high temperatures based on the leaf withering observations. $LaCl_3$ was sprayed on 'Music Box' and 'Galamadrid', which showed better high-temperature tolerance. Physiological response indicators were then analyzed, including chlorophyll, MDA, proline, soluble protein, SOD, and POD.

Total chlorophyll was extracted in 4 mL ethanol from 0.1 g fresh leaves. The total chlorophyll content of leaves was assessed according to Arnon (1949).

For free proline analysis, 0.5 g leaves were soaked in 3% 5-sulfosalicylic acid dehydrate and then boiled for 10 min. Supernatant (2 mL) was mixed with 2 mL acetic acid and 2 mL 2.5% acidic-ninhydrin and then the mixture was put in a boiling water bath for 30 min. After cooling, 4 mL methylbenzene was added and then the solution was centrifuged for 5 min. Free proline content in the upper red supernatant was estimated using the method of Bates et al. (1973).

Soluble protein was isolated from 0.2 g leaves in 5 mL distilled water. After centrifuge, 1 mL supernatant was mixed with 5 mL Coomassie brilliant blue G-250. Protein content in leaves was determined according to Bradford's (1976) method.

For MDA analysis, 0.1 g leaves were ground in 1 mL of 5% trichloroacetic acid. The mixture was centrifuged for 10 min. Supernatant was then mixed with 2 mL 0.67% thiobarbituric acid and then heated at 100 °C for 30 min. MDA content was measured at 532 nm and calculated as described by Heath and Packer (1968).

For enzyme extraction, 0.2 g leaves were frozen in liquid nitrogen and then ground in 2 mL solution containing a 50 mM phosphate buffer (SOD pH 7.8, POD pH 5.5). SOD activity was analyzed by measuring each plant's ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Beyer and Fridovich (1987). POD activity was determined by measuring the rate change in absorbance at 470 nm using Omran's (1980) method.

All spectrophotometric analyses were conducted on an ultraviolet-visible spectroscopy recording spectrophotometer (ultraviolet-160A; Shimadzu, Kyoto, Japan).

HSF sequence clone and expression analysis. The total RNA was extracted from the leaves of different cultivars using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized with Superscript II (Invitrogen). Specific primers were designed according to the DNA-bound domain JN792932.1, HM185815.1, AM490851.1, X82943.1, HM446025.1, considered to be the conserved sequence in HSF of the monocot (forward: 5′-GACGTWCSAGATGGTGRASGAY-3′; reverse: 5′- CTGGAGAARTTGBHGTGCTTGAA-3′). Polymerase chain reaction (PCR) procedures were as follows: 95 °C for 5 min followed by 32 cycles of 94 °C for 30 s, 60 °C for 30 s, and

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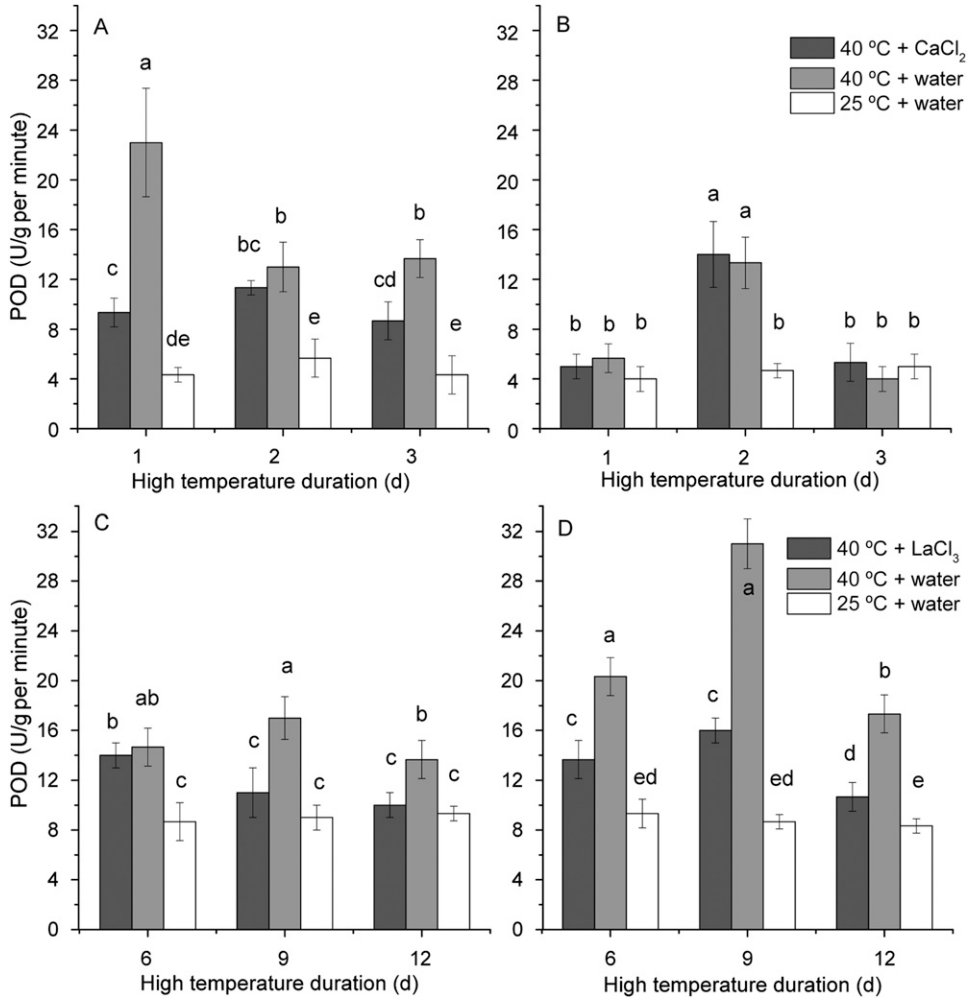

Fig. 4. Peroxidase (POD) content in the leaves of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Potted plants were treated under a high-temperature stress of 40/30 °C (day/night) and sprayed with 100 mL distilled water (40 °C + water), 20 mM $CaCl_2$ (40 °C + $CaCl_2$), or 20 mM $LaCl_3$ (40 °C + $LaCl_3$) everyday. Plants incubated in 25/20 °C (day/night) and supplied with 100 mL distilled water everyday served as the control (25 °C + water). 'Gold Boy' and 'Royal Crusades' were sampled at 1, 2, and 3 d. 'Music Box' and 'Galamadrid' were sampled at 6, 9, and 12 d. The x-axis shows the duration of high-temperature duration by days. Values represent the mean ± SE (n = 3) per treatment. Different letters indicate significant differences at $P < 0.05$ levels.

72 °C for 1 min and then 72 °C for 10 min. After testing with agarose gel electrophoresis, the PCR products were ligated into the pEASY-T1vector (TransGen Biotech, Beijing, China) for sequencing. Obtained sequences were identified through the BLAST program on the web site of the National Center for Biotechnology Information (NCBI, Bethesda, MD).

After confirming the HSF sequence, the gene expressions were analyzed with real-time PCR (RT-PCR). Specific primers for RT-PCR were designed with Primer 5 software (forward: 5′- GACGTGATATCGTGGGGGG-3′; reverse: 5′- AGT GCTTGAAGTGCGCCG-3′). First-stand cDNA was synthesized with the PrimeScript first Strand cDNA Synthesis Kit (TaKaRa, Aomori, Japan). Real-time PCR was performed on a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA) with SYBR Premix Fx Taq II Mix (TaKaRa). The following PCR program was used: 95 °C for 30 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Each PCR was carried out in triplicate. The actin gene was used as an endogenous control and primers were designed with Primer 5 software (forward: 5′- CTTCCCC ATGCTATCCTCCGA-3; reverse: 5′- CCTGACAATTTCCCGCTC TGC-3′). Data were calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis. Reported data are the means of the three replicates. The data collection for each cultivar was designed as a completely randomized experiment. Statistical analyses were performed by variance using SAS (Version 8.0; SAS Institute, Cary, NC) software. Duncan's multiple range test at $P < 0.05$ was applied to compare significant differences between treatments.

Results

Evaluation of the high-temperature stress on four *I. germanica* cultivars

Results showed that high temperature has an extreme influence on these four *I. germanica* cultivars. Results also showed that 100% of the leaves withered with different extent after 2 to 3 d of high-temperature stress for 'Gold Boy' and 'Royal Crusades' and 9 d of stress for 'Music Box' and 'Galamadrid', detailed in Figure 1.

Different cultivars showed different high-temperature sensitivity in terms of the speed of leaf withering. Leave of both 'Gold Boy' and 'Royal Crusades' showed withering on Day 1 of high-temperature stress. Withering began at the tips of the leaves when high-temperature stress was introduced to the plant and gradually extended to the basal area as stress prolonged. All the leaves of the 'Gold Boy' withered by Day 3 of high-temperature stress, and more than 50% of the leaf area withered on Day 4, shown in Figure 1A. 'Royal Crusades' was even more sensitive to high temperature, because 100% of the leaves withered more than 50% by Day 2 of high-temperature stress, shown in Figure 1B. The leaves of 'Music Box' and 'Galamadrid' showed markedly slower withering, where 100% of leaves withered after 9 d, shown in Figure 1C–D, and had not reached 50% withered leaf area until Day 13 of stress.

Physiological response of different *I. germanica* cultivars to high-temperature stress

Total chlorophyll content. Results showed that high-temperature stress was correlated with decreased total chlorophyll content in the four cultivars. For 'Gold Boy', $CaCl_2$ treatment significantly inhibited chlorophyll decrease on Day 3 of high-temperature stress, whereas for 'Royal Crusades', a significant difference occurred on Day 2 of stress, detailed in Figure 2A–B.

'Music Box' and 'Galamadrid' had higher chlorophyll concentrations than 'Gold Boy' and 'Royal Crusades' under

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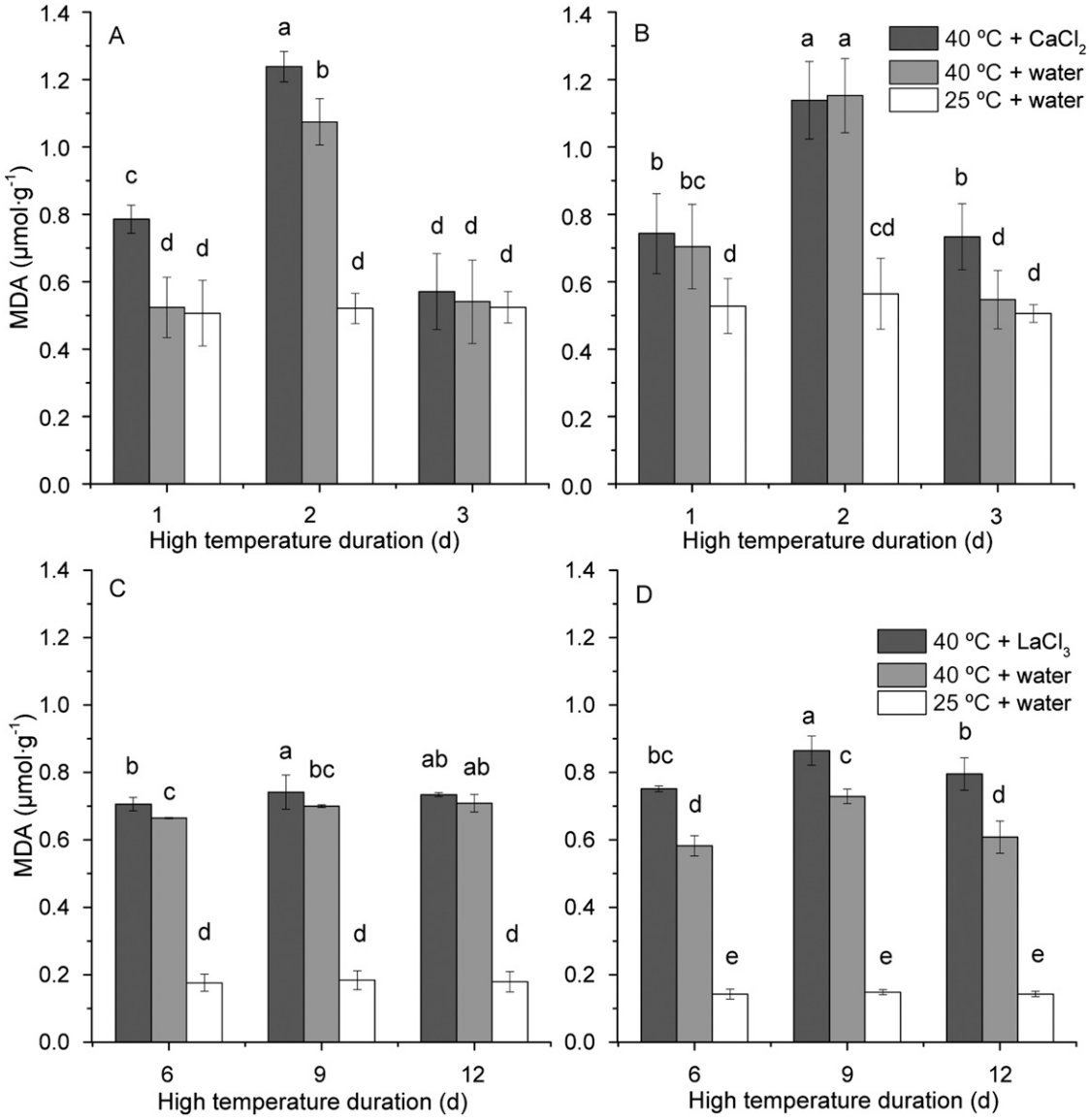

Fig. 5. Malondialdehyde (MDA) content in the leaves of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Potted plants were treated under a high-temperature stress of 40/30 °C (day/night) and sprayed with 100 mL distilled water (40 °C + water), 20 mM $CaCl_2$ (40 °C + $CaCl_2$), or 20 mM $LaCl_3$ (40 °C + $LaCl_3$) everyday. Plants incubated in 25/20 °C (day/night) and supplied with 100 mL distilled water everyday served as the control (25 °C + water). 'Gold Boy' and 'Royal Crusades' were sampled at 1, 2, and 3 d. 'Music Box' and 'Galamadrid' were sampled at 6, 9, and 12 d. The x-axis shows the duration of high-temperature duration by days. Values represent the mean ± SE (n = 3) per treatment. Different letters indicate significant differences at $P < 0.05$ levels.

25 °C. Under high-temperature stress, chlorophyll in the leaves also gradually lessened to a significant level of loss by Day 6 of stress. Moreover, leaves sprayed with $LaCl_3$ showed faster chlorophyll decreases than those sprayed with distilled water, shown in Figure 2C–D.

SOD and POD activity. High-temperature stress was found to increase SOD activity in all four cultivars, shown in Figure 3. $CaCl_2$ treatment resulted in slightly higher SOD activity than distilled water for 'Gold Boy' and 'Royal Crusades' from Day 1 of high-temperature stress. For 'Music Box' and 'Galamadrid', $LaCl_3$ spraying showed less SOD accumulation than water after 6 d of stress.

POD was not significantly affected by $CaCl_2$ compared with water treatment for 'Gold Boy' or 'Royal Crusades', detailed in Figure 4A–B. Water-treated leaves of 'Gold Boy' showed the highest POD activity on Day 1 of high-temperature stress, then gradually decreased under prolonged stress. Nevertheless, $LaCl_3$-sprayed leaves of 'Music Box' and 'Galamadrid' catalyzed significantly less POD than water-sprayed leaves, shown in Figure 4C–D.

MDA content. MDA is considered an indicator of cell membrane lipid peroxidation. In this study, the MDA levels of 'Gold Boy' and 'Royal Crusades' under a controlled temperature (25/20 °C) were similar to that ≈0.5 μmol·g^{-1}. Results showed that under high-temperature stress, MDA significantly increased. In fact, it had more than doubled by Day 2 but decreased to the control level on Day 3 (Fig. 5A–B). $CaCl_2$ treatment showed a slightly higher MDA content than distilled water treatment but did not reach the significance level of 'Royal Crusades'.

For 'Music Box' and 'Galamadrid', MDA remained at a relatively low level, near 0.2 μmol·g^{-1} in the normal temperature. High-temperature stress also stimulated MDA accumulation in leaves over 300%, as shown in Figure 5C–D. MDA

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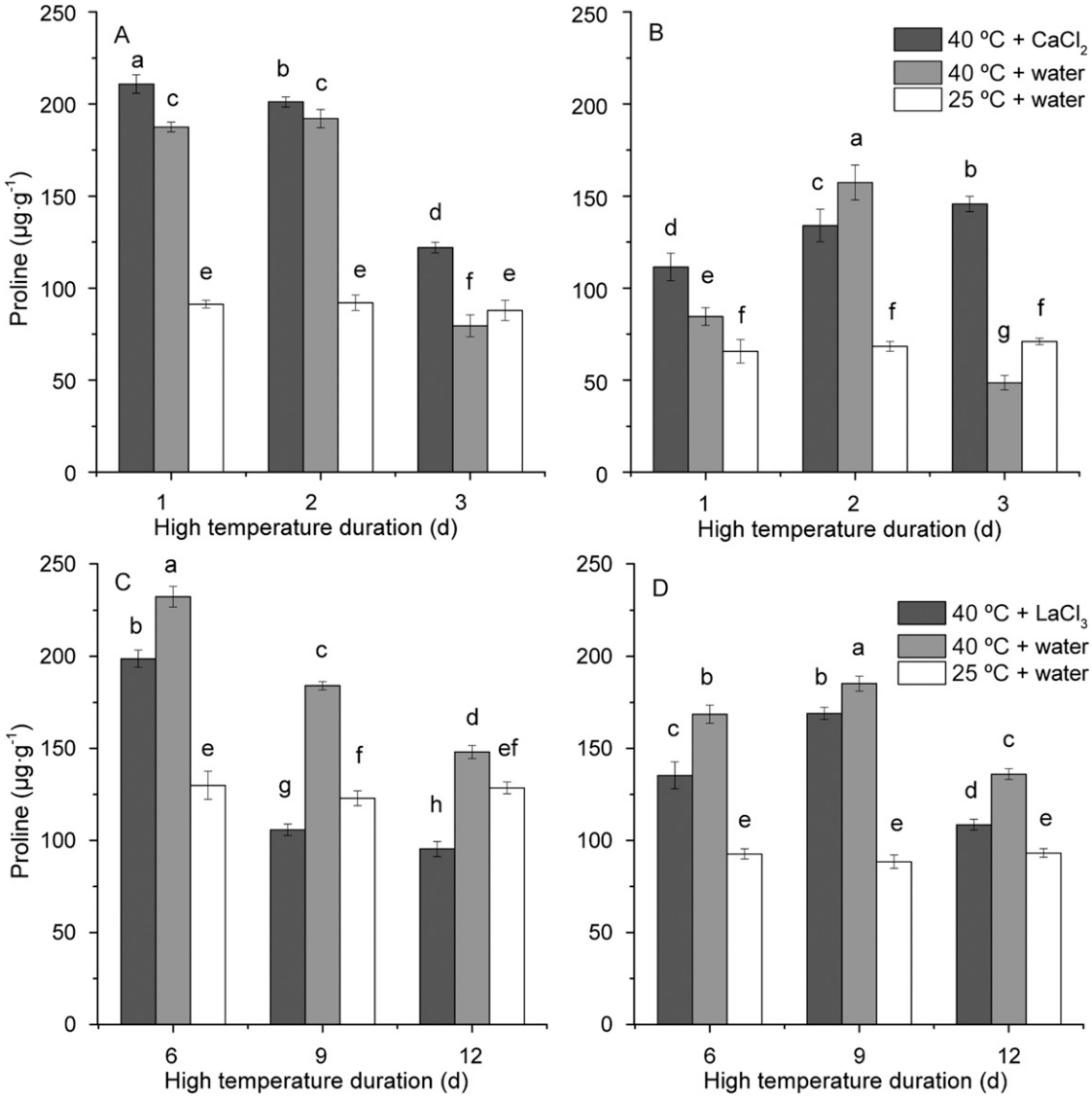

Fig. 6. Proline content in the leaves of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Potted plants were treated under a high-temperature stress of 40/30 °C (day/night) and sprayed with 100 mL distilled water (40 °C + water), 20 mM $CaCl_2$ (40 °C + $CaCl_2$), or 20 mM $LaCl_3$ (40 °C + $LaCl_3$) everyday. Plants incubated in 25/20 °C (day/night) and supplied with 100 mL distilled water everyday served as the control (25 °C + water). 'Gold Boy' and 'Royal Crusades' were sampled at 1, 2, and 3 d. 'Music Box' and 'Galamadrid' were sampled at 6, 9, and 12 d. The x-axis shows the duration of high-temperature duration by days. Values represent the mean ± SE (n = 3) per treatment. Different letters indicate significant differences at $P < 0.05$ levels.

concentration was found to be significantly higher with $LaCl_3$ treatment ($P < 0.05$) compared with distilled water treatment.

Proline content. Proline content increased significantly for all treatments under high-temperature stress relative to the control, as shown in Figure 6. Results showed that the proline in 'Gold Boy' more than doubled as compared with the control on Day 1 of stress and decreased to the normal level on Day 3. $CaCl_2$-treated leaves showed higher proline contents than water-treated leaves (Fig. 6A). For 'Royal Crusades', proline gradually increased in $CaCl_2$-sprayed leaves and remained at a relatively high level compared with water-sprayed leaves, then dropped below the control on Day 3 of stress, detailed in Figure 6B.

Under the control temperature, 'Music Box' showed higher proline content than 'Galamadrid'. $LaCl_3$ treatment was linked to lower proline content than water treatment ($P < 0.05$). For 'Music Box', proline gradually declined in the $LaCl_3$-treated leaves on Day 6 of stress (Fig. 6C), whereas for 'Galamadrid', proline content reached its highest level on Day 9 of stress and then began to drop, shown in Figure 6D.

Soluble protein content. Soluble protein content in the leaves of these four cultivars were significantly affected by both high temperature and $CaCl_2$ or $LaCl_3$ application ($P < 0.05$). 'Gold Boy' and 'Royal Crusades' showed the highest soluble protein concentration on Day 1 of stress but greatly declined by Day 2, shown in Figure 7A–B. $CaCl_2$-treated leaves of 'Gold Boy' had higher protein contents than distilled water-treated leaves on Day 1, whereas leaves of 'Royal Crusades' treated with water formed more protein than those treated with $CaCl_2$ by Day 2 of stress.

$LaCl_3$ treatments were found to cause less soluble protein accumulation in the leaves of 'Music Box' and 'Galamadrid' than water treatments, detailed in Figure 7C–D. Protein gradually decreased for 'Music Box' from Day 6 of stress, whereas for 'Galamadrid', it reached its highest level on Day 9 and then declined.

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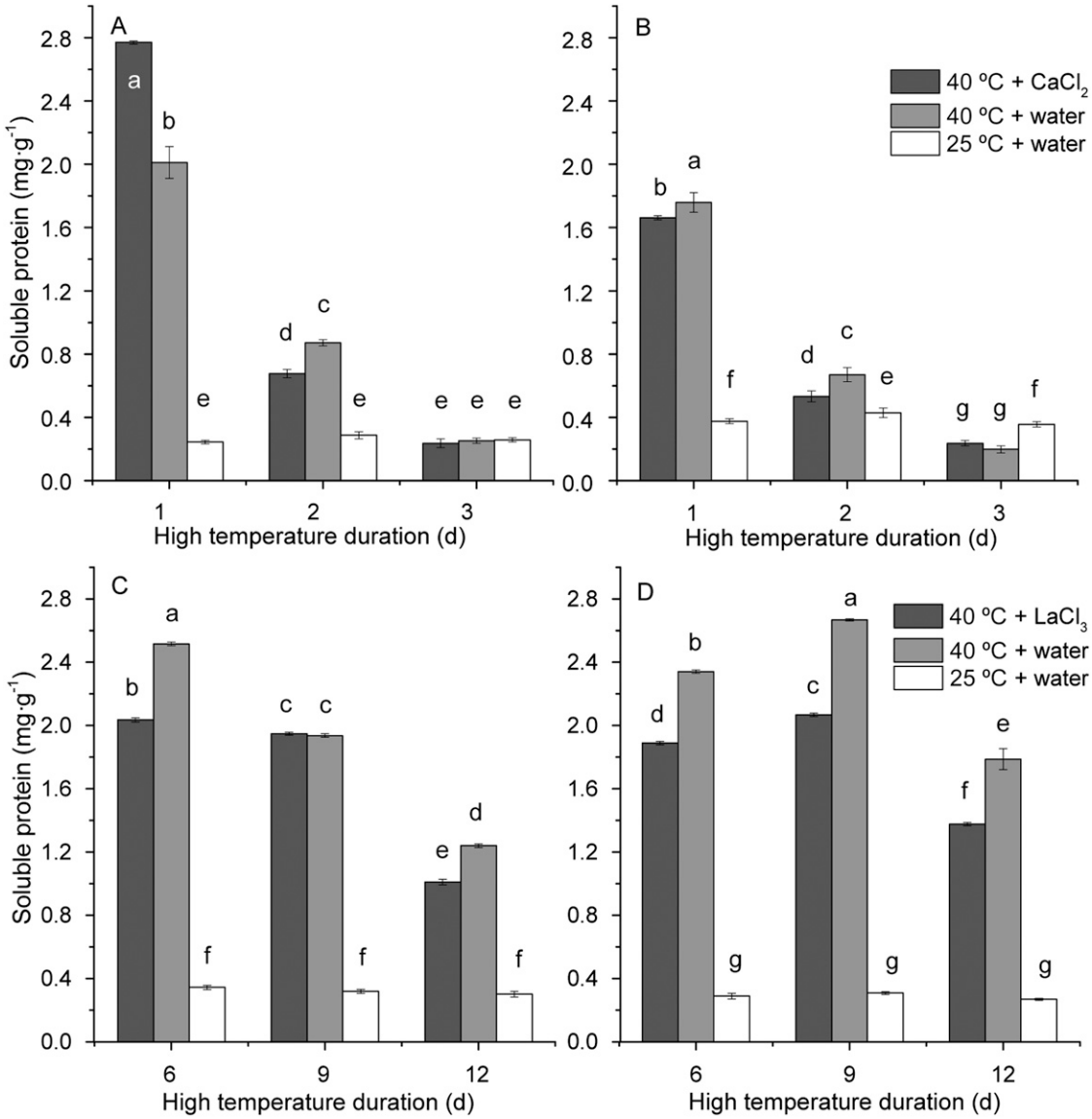

Fig. 7. Soluble protein content in the leaves of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Potted plants were treated under a high-temperature stress of 40/30 °C (day/night) and sprayed with 100 mL distilled water (40 °C + water), 20 mM $CaCl_2$ (40 °C + $CaCl_2$), or 20 mM $LaCl_3$ (40 °C + $LaCl_3$) everyday. Plants incubated in 25/20 °C (day/night) and supplied with 100 mL distilled water everyday served as the control (25 °C + water). 'Gold Boy' and 'Royal Crusades' were sampled at 1, 2, and 3 d. 'Music Box' and 'Galamadrid' were sampled at 6, 9, and 12 d. The x-axis shows the duration of high-temperature duration by days. Values represent the mean ± SE (n = 3) per treatment. Different letters indicate significant differences at $P < 0.05$ levels.

Effects of $CaCl_2$ and $LaCl_3$ on the expression of the *I. germanica* HSF gene under high-temperature stress

To analyze whether the calcium signal is involved in the regulation of HSF expression under high-temperature stress, it was necessary to clone the HSF gene of *I. germanica*. Doing so revealed how the gene changed its expression level when treated with either a Ca^{2+} or ion blocker. Sequencing from the conserved domain of the HSF gene was isolated from the high-temperature-treated leaves of four cultivars, designated GbHsf, RcHsf, MbHsf, and GaHsf for 'Gold Boy', 'Royal Crusades', 'Music Box', and 'Galamadrid', respectively. Sequence analysis using the online NCBI blast program revealed that these partial sequences belonged to the HSF DNA-biding superfamily, considered to be the conserved domain of the HSF group. Details are shown in Figure 8.

To investigate the effect of $CaCl_2$ and $LaCl_3$ on the expression of HSF at 40 °C, mRNA levels of GbHsf, RcHsf, MbHsf, and GaHsf were quantified using quantitative RT-PCR; results are as shown in Figure 9. Under high temperature, $CaCl_2$ significantly generated the expression of the HSF gene on Day 3 of stress. The GbHsf and RcHsf mRNA levels increased to 2.7 and 4.5 times that of the control, respectively. However, on Day 1 of stress, the increase of HSF expression was more obvious in leaves treated with distilled water, as shown in Figure 9A–B.

The expression pattern of MbHsf and GaHsf was quite different from GbHsf and RcHsf. Regardless of $LaCl_3$ vs. water treatment, HSF levels were significantly higher on Day 6 of high-temperature stress but gradually declined to the control level after that. For 'Music Box', $LaCl_3$-treated leaves showed more than a 2.5-fold increase in MbHsf expression level, whereas water-treated leaves increased 4.5-fold compared with the control on Day 6 of stress, shown in Figure 9C. In 'Galamadrid' leaves, both the $LaCl_3$ and water treatment increased GaHsf

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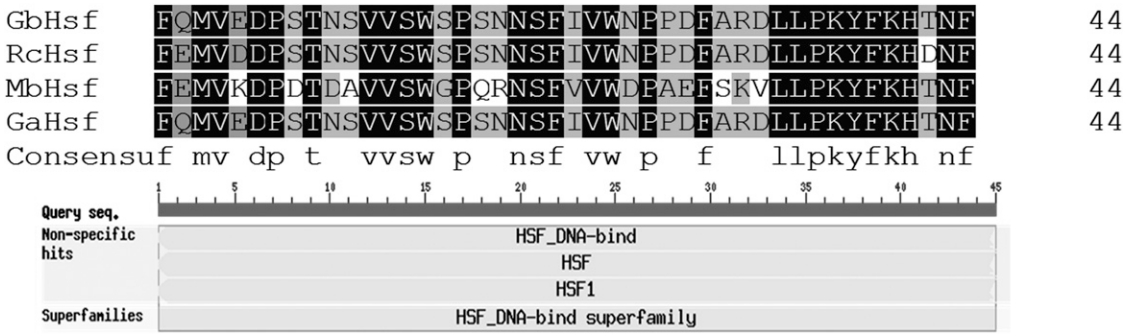

Fig. 8. Alignment of amino acid sequences of heat shock factor (HSF) encoded by partial genes from four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Consensus sequences were identified in the National Center for Biotechnology Information bank as an HSF DNA-biding superfamily: GbHsf ('Gold Boy'), RcHsf ('Royal Crusades'), MbHsf ('Music Box'), GaHsf ('Galamadrid').

expression by four times on Day 6 of stress and gradually declined to the control level afterward, shown in Figure 9D.

Discussion

This study evaluated many parameters related to high-temperature stress treatment, including responses of plants at their morphological, physiological, and molecular levels. Specifically, this work examined controlled high-temperature stress on different *I. germanica* cultivars. In terms of morphological change, high-temperature stress is shown to frequently lead to dehydration and withering in leaves (Simoes-Araujo et al., 2003; Wahid et al., 2007). In these four cultivars, the speed of withering under high-temperature stress was considerably faster than under normal temperature. Similar dehydration in leaves has also been reported in sugarcane (*Saccharum officinarum*) and tomato (Morales et al., 2003; Wahid and Close, 2007). Thus, high temperature is found to be the main reason for large areas of withered leaves in these four cultivars. Moreover, different cultivars showed varying leaf withering speed. The ratio and the area of withered leaves of 'Music Box' and 'Galamadrid' were relatively slower and smaller than those of 'Gold Boy' and 'Royal Crusades', indicating that 'Music Box' and 'Galamadrid' tolerate high temperatures more effectively.

Ca^{2+} has been recognized as the primary intercellular second messenger in plants and is widely used to regulate tolerance to abiotic stress (Gong et al., 1998; Hetherington and Brownlee, 2004). Application of $CaCl_2$ has been found to up-regulate high-temperature tolerance in plants (Liu et al., 2005; Tan et al., 2011; Wang et al., 2009). As described in the results, 100% of the leaves withered within 3 d of high-temperature stress for 'Gold Boy' and 'Royal Crusades', so $CaCl_2$ was planned to applied to these two cultivars to test if Ca^{2+} could improve the high-temperature tolerance as other publications reported. 'Music Box' and 'Galamadrid' were supposed to have better high-temperature tolerance, so $LaCl_3$ was planned to be applied to assay these two cultivars if Ca^{2+} channel blockers could decrease the tolerance.

Results of the $CaCl_2$ effect on 'Gold Boy' and 'Royal Crusades' showed that the degradation rate of leaf chlorophyll content was slightly lower for $CaCl_2$ treatment than for distilled water treatment. $CaCl_2$ treatment also was found to increase proline and soluble protein in leaves under high-temperature stress in accordance with the results of previous studies (Liang et al., 2009; Wahid and Close, 2007). However, it did not provide a notable advantage in improving tolerance to high temperature for 'Gold Boy' or 'Royal Crusades', because physiological parameters assessed in this study showed the significant influence of high-temperature stress for these cultivars, even after treatment. Furthermore, compared with water treatment, $CaCl_2$ treatment was found to cause a slow increase in SOD and POD activity; however, a simultaneous higher MDA accumulation indicates that membrane lipid peroxidation occurred very quickly. To this effect, we speculate that damage from high temperature to these cultivars arises too rapidly for the leaves to efficiently use exogenous Ca^{2+} to catalyze enough antioxidant enzymes and prevent oxidative stress.

This study applied the Ca^{2+} antagonist $LaCl_3$ to 'Music Box' and 'Galamadrid', which have stronger high-temperature tolerance than the other two cultivars studied. Results are consistent with previous reports in that $LaCl_3$ plays a negative role in promoting the activity of antioxidant enzymes and increases proline and soluble protein (Gong et al., 1997; Liang et al., 2009; Snider et al., 2011). Moreover, $LaCl_3$-sprayed leaves were found to accumulate more MDA than leaves that had undergone water treatment. This implies that $LaCl_3$ likely blocked the Ca^{2+} signal in response to high-temperature stress (Graziana et al., 1988; Liang et al., 2009). Results also show that $LaCl_3$ treatments caused faster degradation of chlorophyll content than distilled water treatment. This may be the result of accelerating photo-oxidation or down-regulation of membrane integrity (Coria et al., 1998; Tan et al., 2011). However, 'Music Box' and 'Galamadrid' did not show a dramatic loss of high-temperature resistance with $LaCl_3$. Thus, we presume that $LaCl_3$ may only block parts of the endogenous Ca^{2+} signal pathway that affect the high-temperature response in these cultivars. It is also possible that these two cultivars use an alternative bioprocess to regulate their high-temperature tolerance.

Activation of HSF is an important mechanism of transcriptional regulation for the expression of heat shock protein (Baniwal et al., 2004; Wunderlich et al., 2003). HSF can be induced by Ca^{2+} or inhibited by the Ca^{2+} antagonist (Cao et al., 2013; Mosser et al., 1990; Zhou et al., 2009). This study showed that the application of $CaCl_2$ causes up-regulation of the expression of HSF genes in 'Gold Boy' and 'Royal Crusades' in the later period of leaf damage under high-temperature stress. These results are consistent with the physiological response of 'Gold Boy' and 'Royal Crusades', implying that although Ca^{2+} helped to use certain enzymes, proteins, and genetic molecular mechanisms against high-temperature stress, the process began too late for the plants to compensate for severe injury that had already occurred. Additionally, maintaining HSF transcription at a high level to accumulate

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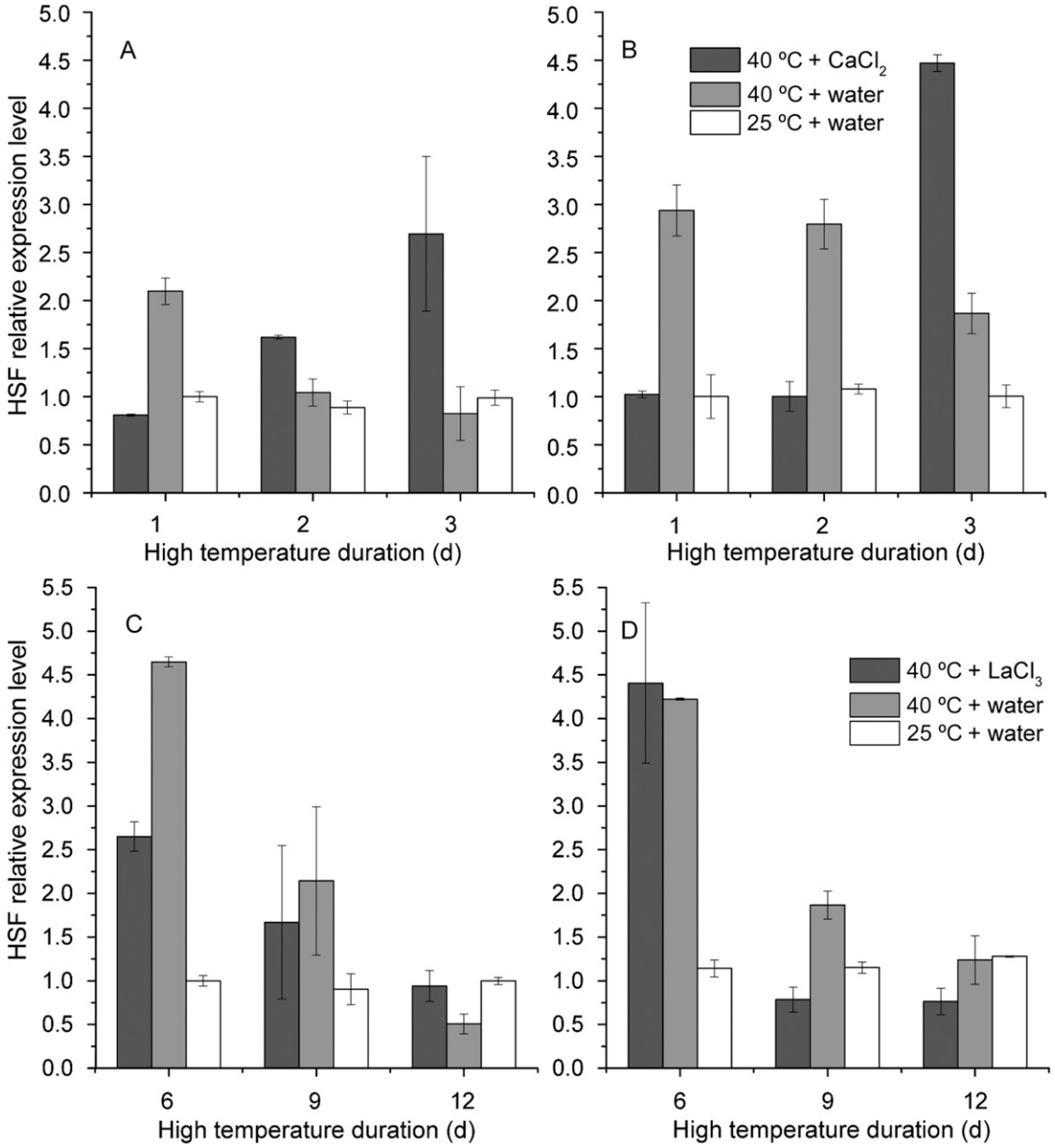

Fig. 9. Heat shock factor (HSF) expression level in the leaves of four *Iris germanica* cultivars [(**A**) Gold Boy, (**B**) Royal Crusades, (**C**) Music Box, (**D**) Galamadrid]. Potted plants were treated under high-temperature stress of 40/30 °C (day/night) and sprayed with 100 mL distilled water (40 °C + water), 20 mM $CaCl_2$ (40 °C + $CaCl_2$), or 20 mM $LaCl_3$ (40 °C + $LaCl_3$) everyday. Plants incubated in 25/20 °C (day/night) and supplied with 100 mL distilled water everyday were used as a control (25 °C + water). 'Gold Boy' and 'Royal Crusades' were sampled at 1, 2, and 3 d. 'Music Box' and 'Galamadrid' were sampled at 6, 9, and 12 d. The x-axis shows the duration of high-temperature duration by days. Error bars represent ± SE of the three replicate samples from which RNA were isolated.

heat shock proteins may incur significant nitrogen costs and have detrimental effects on plants (Amano et al., 2012; Feder and Hofmann, 1999). This could also explain the dramatic decline of soluble protein in the leaves of 'Gold Boy' and 'Royal Crusades' after 2 d of high-temperature stress.

Generally, 'Music Box' and 'Galamadrid' were found to manage high-temperature stress better than the other two cultivars tested. In addition, their HSF genes were down-regulated when $LaCl_3$ was sprayed on leaves compared with leaves sprayed with water. This indicates that Ca^{2+} is probably involved in the HSF transcriptional regulation that enhances high-temperature tolerance. Influx of Ca^{2+} into plant cytoplasm can arise from extracellular sources or an intracellular Ca^{2+} pool (Sanders et al., 2002). Endogenous Ca^{2+} has been found to increase significantly in cells as induced by heat shock in *Drosophila melanogaster*, *Pisum sativum*, and *Nicotiana tabacum* (Biyaseheva et al., 1993; Gong et al., 1998; Stenvenson et al., 1986). We speculate that 'Music Box' and 'Galamadrid' possess a sufficient endogenous Ca^{2+} pool as well as an efficient Ca^{2+} transmission channel to acclimate effectively to high-temperature stress.

Conclusions

High-temperature stress has become a problem that seriously affects the quality and quantity of production of ornamental plants cultivated worldwide. This study demonstrates that high-temperature stress leads to cultivar-specific changes in leaf withering speed, chlorophyll content, antioxidant enzymes,

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MDA content, free proline, soluble protein, and heat shock factor expression in *I. germanica*. The application of $LaCl_3$ in 'Music Box' and 'Galamadrid' proved Ca^{2+} involvement in the regulation of physiological indicators and HSF expression level. Although $CaCl_2$ can manipulate endogenous enzymes, proteins, and genetic molecular mechanisms, this process still occurred too late for 'Gold Boy' and 'Royal Crusades' to compensate for the damage rapidly caused by high-temperature stress. Comparative studies on different Iris cultivars under high temperatures may further clarify the physiological adaptation of different ecological types to facilitate the best-fitting selection of applicable cultivars for cultivation and sale in subtropical China.

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