

## Short-term cold-shock at 1°C induced chilling tolerance in maize seedlings

Hai-Yan Li<sup>1,2</sup>

<sup>1</sup>School of Life Sciences  
Yunnan Normal University  
Kunming, China

<sup>2</sup>School of Environmental and Biological Engineering  
Shenyang University of Chemical Technology  
Shenyang, China  
e-mail: lihayan98@163.com

Chun-Guang Li<sup>1,3</sup>

<sup>3</sup>Department of Industrial Engineering  
Zhengzhou Institute of Aeronautical Industry  
Management  
Zhengzhou, China  
e-mail: lcgstress@163.com

Ming Gong<sup>1</sup>

email:gongming@ynnu.edu.cn

**Abstract**—1°C is normally considered as an extreme chilling stress temperature and will cause severe chilling injury to chilling-sensitive plants. Chilling acclimation of chilling-sensitive plants was usually achieved by exposure to around or above the threshold chilling temperature (about 10°C or 15°C) for several days. In our present work, a cold-shock pretreatment at 1°C for 4 h, followed by a 6-h recovery at 26.5°C, significantly enhanced survival rate of maize seedlings, mitigated electrolyte leakage of primary roots and alleviated vitality loss of mesocotyls under severe chilling stress at 1°C, indicating that short-term cold-shock could induce chilling tolerance of maize seedlings. The cold-shock pretreatment brought out an endogenous hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) peak, enhanced the activities of five antioxidant enzymes guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD) in maize mesocotyls and remained significantly higher activities of these five antioxidant enzymes after chilling stress. These results imply that antioxidant systems play a pivotal role in the cold-shock-induced chilling tolerance of maize seedlings, and H<sub>2</sub>O<sub>2</sub> may be involved in signaling and triggering of this cold-shock-induced chilling tolerance through the mobilization of antioxidant systems.

**Keywords**—cold shock; chilling tolerance; antioxidant enzymes; hydrogen peroxide; maize seedling

### I. INTRODUCTION

Chilling-sensitive plants native to warm regions, such as maize, cotton, soybean, rice, cucumber, tomato and so on, are generally injured at temperatures around or below 10°C to 15°C (threshold chilling temperatures). Visual symptoms of chilling damage in vegetative tissue often include surface lesions and necrotic areas, waterlogged appearance, internal discoloration, wilting, acceleration of senescence, reduction in growth, and eventual death[1].

Cold acclimation is a universal phenomenon in chilling-tolerant plants such as wheat, barley, rye, beet and so on [2]. In addition, chilling hardening or acclimation also can be achieved in some chilling-sensitive plants by

exposure to a moderate low temperature. For example, continuous acclimation of rice seedlings at 20/15°C (day/night) for 15 days, and 10/10°C for 6 days could significantly improve their chilling tolerance at 5°C [3]. Soybean seedlings was acclimated to survive a lower temperature of 8°C by a 5-d exposure to 14/8°C cycles [4]. Three-day-old maize seedlings did not survive chilling stress at 4°C for 7 days unless they were pre-exposed to 14°C for 3 days [5]. Pretreatment of etiolated cucumber seedlings at 12°C for 48 h followed by a warming period at 25°C for 12 h led to their tolerance to subsequent chilling at 2°C [6]. Seven-day-old mung bean seedlings acclimated at 8°C for 36 h showed 97% survival after a 36-h, 4°C chilling stress, compared with 33% survival of non-acclimated plants [7]. Overviewing these results, the acclimation or hardening of chilling-sensitive plants was achieved by exposure to around or above the threshold temperature that caused chilling injury for several days. However, whether chilling acclimation can be achieved by exposure to under the threshold chilling temperature, even as low as 1°C, which was generally known as the temperature of severe chilling injury, hasn't been documented.

In the present study, we found that cold-shock at 1°C for several hours could significantly induce chilling tolerance of maize seedlings and investigated the changes of antioxidant enzyme activities after the cold-shock and chilling stress and involvement of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in signaling and triggering of the cold-shock-induced chilling tolerance.

### II. MATERIALS AND METHODS

#### A. Plant Material

A commercial variety of maize (*Zea mays* L., DaHuang) was used in the present experiments. The seeds were sterilized in 0.1% HgCl<sub>2</sub> for 10 min, and pre-soaked for imbibition in distilled water for 12 h. The soaked seeds were sowed on six layers of wetted filter papers in trays with covers and germinated at 26.5°C in the dark for 60 h. Germination percentage was over 90%.

### B. Cold-shock Pretreatment, Chilling Treatment and Investigation of Survival Percentage

Germinating seedlings were transferred to 1°C in the dark for 2, 4 or 6 h for cold-shock. After the cold-shock pretreatments, the seedlings were cultured at 26.5°C in the dark for 6 h for recovery. The control seedlings were kept at 26.5°C. Afterwards, seedlings with and without cold-shock were transferred to 1°C in the dark for 5 days for chilling treatment. At the end of chilling treatment, the treated seedlings were transferred to normal culture condition at 26.5°C for 8 days with 16 h illumination for recovery and their survival percentage counted. The seedlings which could re-grow and become green during recovery were considered to have survived.

### C. Measurement of Electrolyte Leakage of Roots

During the treatments as described above, 1.0 cm tips of primary roots were cut off, and the leakage percentage of electrolytes from the root tips was measured according to [8].

### D. Assay of Mesocotyl Vitality

The vitality of maize mesocotyls (1 cm section) was measured using triphenyltetrazolium chloride (TTC) according to [8].

### E. Enzyme Assays

Maize mesocotyls (0.5 g) were homogenised in liquid nitrogen and soluble proteins were extracted by the method of [9]. The supernatant was used for the following enzyme assays.

Guaiacol peroxidase (GPX) activity was determined by the method of [10]. Catalase (CAT) activity was assayed by the method of [11]. Ascorbate peroxidase (APX) activity was measured according to the method of [12]. Superoxide dismutase (SOD) activity was determined by the method of [13]. Glutathione reductase (GR) activity was determined by the method of [9]. One unit of activity for GPX, CAT, APX, GR and SOD was defined as the amount of enzyme which caused the formation of 1µmol tetraguaiacol per min, degraded 1µmol H<sub>2</sub>O<sub>2</sub> per min, oxidized 1µmol of ascorbic acid per min, oxidized 1µmol of nicotinamide adenine dinucleotide phosphate (NADPH) per min, inhibited 50% of the photochemical reduction of nitroblue tetrazolium.

Soluble protein content in the extracts for enzyme assay was determined by [14].

### F. Determination of H<sub>2</sub>O<sub>2</sub> Contents

H<sub>2</sub>O<sub>2</sub> content of maize mesocotyls was measured by [15].

## III. RESULTS

### A. Cold-shock-induced chilling tolerance

Chilling stress at 1°C for 5 days led to the death of about 70% of maize seedlings. Cold-shock pretreatment at 1°C for 2, 4 or 6 h, followed by a 6-h recovery at 26.5°C, could significantly increase the survival rate of maize seedlings at 1°C for 5 days (Fig. 1). The 4-h cold-shock pretreatment showed the best effect among these treatments for induction

of chilling tolerance and survival rate of the seedlings cold-shocked for 4 h was enhanced by 103.2% as compared with the non-cold-shock control (Fig. 1).

To compare the difference of response of the cold-shocked and non-cold-shocked seedlings to chilling stress at 1°C, we investigated the change of their survival rate during the chilling stress for 7 days. As shown in Fig. 2, the seedlings had no death in first 2 days of the chilling stress. With the prolonged chilling time, the survival rate declined sharply from 3<sup>th</sup> to 6<sup>th</sup> day and finally the seedlings died completely after 7 days' chilling stress. The cold-shocked seedlings showed significantly higher survival rate than the non-cold-shock control during chilling stress from 3<sup>th</sup> to 6<sup>th</sup> day.

To substantiate the above survival rate data, we also measured plasma membrane damage and tissue vitality loss of the cold-shocked and non-cold-shocked maize seedlings under the chilling stress. Environmental stresses primarily damage membrane systems and lead to leakage of electrolytes from plant cells [8].

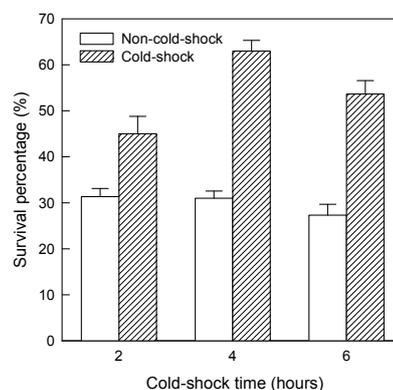


Fig. 1. Effect of cold-shock pretreatment on survival percentage of maize seedlings

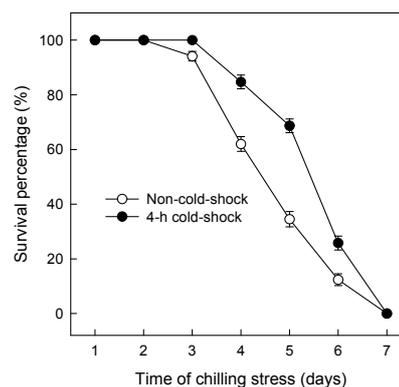


Fig. 2. Change of survival percentage of cold-shocked and non-cold-shocked maize seedlings during chilling stress.

In the present experiments, the maize seedlings significantly increased their leakage of electrolytes from root tips after exposure to chilling stress (Fig. 3A). A cold-shock pretreatment remarkably reduced the electrolyte leakage during chilling stress as compared with the control without cold-shock pretreatment, and this difference

demonstrated the most obvious during the middle stage of the chilling stress (Fig. 3A).

TTC reduction activity is also widely used to evaluate plant's resistance to environmental stresses[8]. In the present experiments, the vitality of maize mesocotyls declined sharply following chilling stress (Fig. 3B). The cold-shock pretreatment significantly alleviated the loss of mesocotyl vitality during chilling stress as compared with the control without cold-shock pretreatment (Fig. 3B).

### B. Cold-shock-induced H<sub>2</sub>O<sub>2</sub> Change

The change of endogenous H<sub>2</sub>O<sub>2</sub> content in maize mesocotyls was detected during cold-shock pretreatment 1°C. As shown in Fig. 4, H<sub>2</sub>O<sub>2</sub> content increased markedly during the first 4-h of cold-shock treatment, then declined gradually.

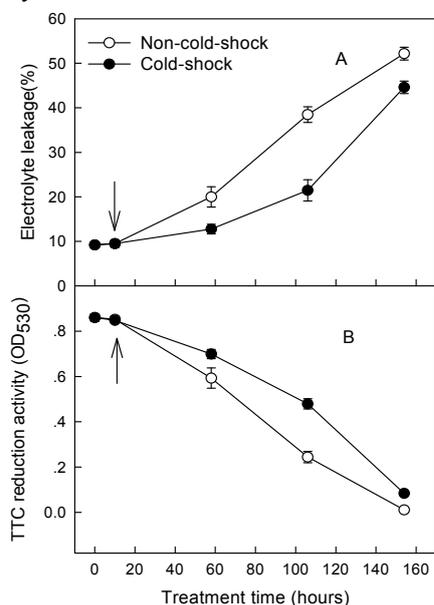


Fig. 3. Effect of cold-shock pretreatment on electrolyte leakage of root tips (A) and mesocotyl vitality (B) of maize seedlings during chilling stress. The 0-h points represent the result of seedlings before cold-shocked and 10-h points (the arrows) represent the result of the seedlings exposed to 1°C for 4 h and recovered for 6 h, namely the 0-h point of chilling stress at 1°C for 6 days. Other points respectively represent the results of the seedlings chilled at 1°C for 2, 4 and 6 days.

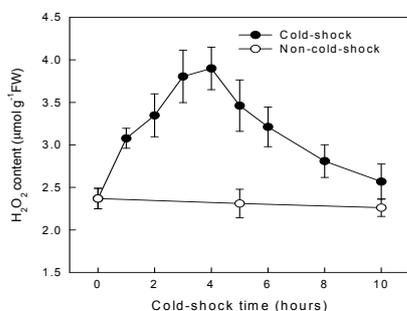


Fig. 4. Change of H<sub>2</sub>O<sub>2</sub> content of maize mesocotyls during cold-shock pretreatment at 1°C

### C. Effect of Cold-shock Pretreatment on Antioxidant Enzymes

Numerous studies showed that antioxidant enzymes are involved in the regulation of responses of plants to environmental stresses[5,9]. So we investigated the changes of activities of the five antioxidant enzymes GPX, CAT, APX, GR and SOD in maize seedlings after pretreatment (the cold-shock and then recovery) and chilling stress. As shown in Table 1, pretreatment increased the activities of the five enzymes in contrast to non-cold-shock control. When the seedlings with and without cold-shock were treated at 1°C for 5 days, the cold-shocked seedlings totally remained significantly higher activities of these five antioxidant enzymes than control.

TABLE 1 EFFECT OF COLD-SHOCK PRETREATMENT ON ANTIOXIDANT ENZYMES

Enzyme activity (Units mg <sup>-1</sup> protein)	After Pretreatment		Chilling Stress For 5 Days	
	Cold shock	Control	Cold shock	Control
GPX	81.23± 1.23	57.59± 2.61	57.24± 2.21	49.35± 1.67
CAT	48.59± 3.21	32.63± 1.98	31.76± 1.15	20.98± 1.79
APX	4.23± 0.88	3.78± 0.59	3.85± 0.26	3.12± 0.17
GR	30.05± 1.62	21.97± 1.03	27.85± 2.12	18.26± 1.21
SOD	53.38± 2.56	42.21± 2.04	57.12± 2.46	50.04± 1.37

## IV. DISCUSSION

1°C is normally considered as a extreme chilling stress temperature and will cause severe chilling injury to chilling-sensitive plants. Chilling acclimation of chilling-sensitive plants was usually achieved by exposure to around or above the threshold chilling temperature (about 10 to 15°C) for several days [5]. However, the present results showed that a short-term cold-shock at the temperature as low as 1°C for 2 to 6 h plus a 6-h recovery could significantly enhance survival rate of maize seedlings (Figs 1, 2), alleviated the electrolyte leakage from roots and vitality loss in mesocotyls (Fig. 3), indicating that the cold-shock at 1°C indeed improved chilling tolerance of the maize seedlings.

The mechanism of cold-shock-induced chilling tolerance at the temperature as low as 1°C in chilling-sensitive plants, to our best knowledge, is not well documented. But it was well known that oxidative stress is a major component of chilling stress in chilling-sensitive plants [16]. Many studies have linked chilling tolerance to antioxidant capacity in chilling-sensitive plants. Elevated levels of SOD, APX, CAT in cucumber seedling radicles appear to be correlated with the development of chilling tolerance [17]. When maize seedlings were acclimated to low temperature, antioxidant enzyme activities were substantially increased[5]. In the present experiments, our results showed that 1°C cold-shock pretreatment, which induced chilling tolerance in maize seedlings (Figs 1, 2 and 3), enhanced activities of the five antioxidant enzymes GPX, CAT, APX, GR and SOD (Table 1). The increase could make the

cold-shocked maize seedlings retain higher activities of antioxidant enzymes after chilling stress (Table 1). This suggested that in the cold-shocked seedlings, improvement of chilling tolerance was partly due to an enhanced antioxidant enzyme system.

It is now clear that the active oxygen species  $H_2O_2$  is not only a toxic cellular metabolite but also functions as a signaling molecule that mediates responses to various stimuli in both plant and animal cells [18]. Our results showed that the cold-shock pretreatment, which induced chilling tolerance in maize seedlings (Figs 1, 2), brought out an endogenous  $H_2O_2$  peak (Fig. 4). Previous research showed that exogenous  $H_2O_2$  pretreatment could elevate activities of the antioxidant enzymes [5]. In addition, exogenous  $H_2O_2$  pretreatment also improved chilling tolerance in maize seedlings [5,8]. All these may indicate that early accumulation of  $H_2O_2$  during the cold-shock pretreatment could play a key role as a signaling molecular to trigger antioxidant protective mechanisms and finally improves chilling tolerance in maize seedlings.

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#### REFERENCES

- [1] M.E. Saltveit, "Chilling injury is reduced in cucumber and rice seedlings and in tomato pericarp discs by heat-shocks applied after chilling," *Postharv. Biol and Technol*, vol. 21, January 2001, pp. 169-177, doi:10.1016/S0925-5214(00)00132-0.
- [2] Z. Xin and J. Browse, "Cold comfort farm: the acclimation of plants to freezing temperatures," *Plant. Cell. Environ*, vol. 23, September 2000, pp. 893-902, doi: 10.1046/j.1365-3040.2000.00611.x.
- [3] M. Gong, Y.L. Liu and P.R. Zhu, "Physiology effects of chill and drought hardening on cold resistance of clones of hybrid japonica rice," *J. Nanjing. Agri. Univ. China*, vol. 12, March 1989, pp. 22-27, doi: cnki:ISSN:1000-2030.0.1989-01-003.
- [4] M. Cabané, P. Calvet, P. Vincens and A.M. Boudet, "Characterization of chilling-acclimation-related proteins in soybean and identification of one as a member of the heat shock protein (HSP 70) family," *Planta*, vol. 190, June 1993, pp. 346-353, doi: 10.1007/BF00196963.
- [5] T.K. Prasad, M.D. Anderson, B.A. Martin and C.R. Stewart, "Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide," *Plant Cell*, vol. 6, January 1994, pp. 65-74, doi: 10.1105/tpc.6.1.65.
- [6] A. Erez, E. Cohen and C. Frenkel, "Oxygen-mediated cold-acclimation in cucumber (*Cucumis sativus*) seedlings," *Physiol. Plant*, vol. 115, August 2002, pp. 541-549, doi: 10.1034/j.1399-3054.2002.1150408.x.
- [7] C.W. Yu, T.M. Murphy, W.W. Sung and C.H. Lin, " $H_2O_2$  treatment induces glutathione accumulation and chilling tolerance in mung bean," *Funct. Plant. Biol*, vol. 29, September 2002, pp. 1081-1087, doi: 10.1071/PP01264.
- [8] M. Gong, B. Chen, Z.G. Li and L.H. Guo, "Heat-shock-induced cross adaptation to heat, chilling, drought and salt stress in maize seedlings and involvement of  $H_2O_2$ ," *J. Plant. Physiol*, vol. 158, September 2001, pp. 1125-1130, doi:10.1078/0176-1617-00327.
- [9] Knörzner, J. Burner and P. Böger, "Alterations in the antioxidative system of suspension-cultured soybean cells (*Glycine max*) induced by oxidative stress," *Physiol. Plant*, vol. 97, August 1996, pp. 388-396, doi: 10.1034/j.1399-3054.1996.970225.x.
- [10] B. Chance and A.C. Maehly, "Assay of catalases and peroxidases," *Methods. Enzymol*, vol. 2, December 1955, pp. 764-775, doi: 10.1016/S0076-6879(55)02300-8.
- [11] H. Aebi, "Catalase in vitro," *Methods. Enzymol*, vol. 105, March 1984, pp. 121-126, doi:10.1016/S0076-6879(84)05016-3.
- [12] Y. Nakano and K. Asada, "Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts," *Plant. Cell. Physiol*, vol. 22, August 1981, pp. 867-880, Retrieved from <http://pcp.oxfordjournals.org/>
- [13] C.N. Giannopolitis and S.K. Ries, "Superoxide dismutase. I. Occurrence in higher plants," *Plant. Physiol*, vol. 59, pp. 309-314, February 1977. doi:10.1104/pp.59.2.309.
- [14] M.M. Bradford, "A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem*, vol. 72, May 1976, pp. 248-254, doi:10.1016/0003-2697(76)90527-3.
- [15] T. Brennan and C. Frenkel, "Involvement of hydrogen peroxide in the regulation of senescence in pear," *Plant. Physiol*, vol. 59, March 1977, pp. 411-416, doi:10.1104/pp.59.3.411.
- [16] R.G. Pinhero, M.V. Rao, G. Paliyath, D.P. Murr and R.A. Fletcher, "Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings," *Plant. Physiol*, vol. 114, June 1997, pp. 695-704, Retrieved from <http://www.plantphysiol.org/>
- [17] H.M. Kang and M.E. Saltveit, "Activity of enzymatic antioxidant defense systems in chilled and heat shocked cucumber seedlings radicles," *Physiol. Plant*, vol. 113, December 2001, pp. 548-556, doi:10.1034/j.1399-3054.2001.1130414.x.
- [18] S.J. Neill, R. Desikan, A. Clarke, R.D. Hurst and J.T. Hancock, "Hydrogen peroxide and nitric oxide as signalling molecules in plants," *J. Exp. Bot*, vol. 53, May 2002, pp. 1237-1247, doi: 10.1093/jexbot/53.372.1237