Repetition of Hydrogen Peroxide Treatment Induces a Chilling Tolerance Comparable to Cold Acclimation in Mung Bean

Shu Hsien Hung
Department of Bioindustry Technology, Da Yeh University, Changhua, Taiwan

Chun Chi Wang
Department of Molecular Biotechnology, Da Yeh University, Changhua, Taiwan

Sergei Veselinov Ivanov and Vera Alexieva
Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Bl. 21, 1113 Sofia, Bulgaria

Chih Wen Yu1
Department of Molecular Biotechnology, Da Yeh University, Changhua, Taiwan

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Abstract. Mung bean seedlings (Vigna radiata L.) of the cultivar Tainan No. 5 (a chilling-sensitive cultivar) pretreated with multiple sprays of 200 mM H2O2 showed a tolerance to chilling at 4 °C for 36 h, measured by electrolyte leakage, that was greater than that induced by a single treatment and similar to that induced by cold-acclimation at 10 °C for 48 h. Two H2O2 treatments at an interval of 3 h gave the optimum chilling tolerance. Tolerance induced by H2O2 could be distinguished from that induced by acclimation at 10 °C according to length at 4 °C and corresponding electrolyte leakage. Chilling tolerance induced by H2O2 depended on accumulation of glutathione (GSH), which could be significantly reversed by pretreatment with buthionine sulfoximine (BSO). In contrast, tolerance induced by incubation at 10 °C for 48 h in light was neither accompanied by accumulation of GSH nor reversed by BSO, suggesting that there are at least two independent mechanisms of developing chilling tolerance. Chilling tolerance of both cold-acclimated and H2O2-treated seedlings was decreased by ethyleneglycol-bis(aminohethyl ether)-N,N′-tetra-acetic acid (EGTA) but not by ruthenium red, indicating that the influx of Ca2+ from extracellular, but not intracellular, pools is an important signal in the induction of tolerance. In confirmation, sprays of Ca2+ could be substituted for H2O2.

Environmental stress causes considerable losses in productivity of many crops. Among various stresses, low temperature is one of the most crucial signals affecting plant growth and even leading to death (Sung et al., 2003; Veal et al., 2007). Extensive study on oxidative stress has demonstrated that exposure of plants to low temperature always induces the overproduction of reactive oxygen species (ROS), such as superoxide radical (O2·−), H2O2, and hydroxyl radical (HO·) in plant cells (Hung et al., 2005). ROS are highly reactive to membrane lipids, protein, and DNA; they are believed to be one of the major contributing factors to chilling injuries (CIs) and to cause rapid cellular damage (Hariyadi and Parkin, 1993; O’Kane et al., 1996; Prasad, 1996). When plants are exposed to low temperature, electron-transport chains tend to form O2·−, which dismutates to form H2O2. Furthermore, in chloroplasts, low temperature limits the dark reactions, thus limiting the supply of NADPH and favoring reduction of O2 by photosystem II. Therefore, exposure to low temperature in combination with high light intensity leads to more serious damage in plants (Allen and Ort, 2001). In mitochondria, inhibition of ATP formation or electron flow through cytochrome b stimulates O2·− formation by complex I and by ubiquinone (Elstner, 1991).

Plants have evolved both enzymatic and nonenzymatic mechanisms to scavenge the ROS rapidly evolved under low-temperature stress (Apel and Hirt, 2004; Scandalios, 1993). Among the antioxidant mechanisms, the ascorbic acid (AsA)–GSH cycle is a key component for elimination of ROS, especially H2O2 (Kingston-Smith and Foyer, 2000; Noctor et al., 2002). In the AsA–GSH cycle, AsA reduces both O2·− and H2O2. In turn, the crucial antioxidant, GSH, reduces dehydroascorbate to regenerate AsA; meanwhile, GSH itself is oxidized to form GSH disulfide (GSSG). NADPH, catalyzed by glutathione reductase (GR), then reduces GSSG to regenerate GSH (Kocsy et al., 2000a, 2000b, 2001). Therefore, inhibition of GSH synthesis by a specific inhibitor, BSO, could dramatically decrease the chilling tolerance of mung bean seedlings and maize (Zea mays L.) (Kocsy et al., 2002; Kocsy et al., 2000b; Marchi et al., 2002, 2003). Experimental evidence also indicates that the level and redox state of GSH might serve as indicators of plant responses to environmental stresses such as chilling (Foyer et al., 1997; Kocsy et al., 1998; Tausz et al., 2004). However, how plants sense low temperature and then transmit a precise signal to eventually elevate the cellular GSH levels is still far from clear.

According to our present understanding of signal transduction in plant cells, (Ca2+)cyt plays a pivotal role. The second...
messengers Ca²⁺ triggers cellular changes in response to many different signals (e.g., light, hormones, touch, cold, fungal elicitors, and even H₂O₂) (Knight, 2000; Knight et al., 1996; Sanders et al., 1999). It was reported that transient increases in (Ca²⁺)ₜₚ levels could be evoked by cold treatment in Arabidopsis [Arabidopsis thaliana (L.) Heynh.] (Knight et al., 1996; Lewis et al., 1997; Polisensky and Braam, 1996; Sung et al., 2003). An influx of extracellular Ca²⁺ seems to play a major role in the low-temperature response, and an intracellular Ca²⁺ source might also be involved (Polisensky and Braam, 1996; Rentel and Knight, 2004). Evidence also indicated that H₂O₂-activated Ca²⁺ channels mediated both the influx of Ca²⁺ in protoplasts and increases in (Ca²⁺)ₓₜ in intact guard cells, thus leading to closure of stomata of Arabidopsis (Pei et al., 2000). Interestingly, in addition to serving as a link in a signaling cascade, fluctuation of (Ca²⁺)ₓₜ could be one of the mechanisms that lead plants to memorize what they have suffered (Knight et al., 1996). This inference comes from the observation that Arabidopsis treated with either sublethal cold or H₂O₂ modifies its Ca²⁺ signal in response to subsequent cold stress as compared with untreated control (Knight et al., 1996). On the basis of these findings, it was then proposed that the “calcium memory” is an important mechanism for plants adapt to environmental changes.

In Taiwan, the temperature normally ranges between 17 and 27 °C, but occasionally it may decline to 10 °C or lower and remain for a few days. Hence, it is imperative to develop a simple and reliable method to decrease the agricultural losses due to chilling. In this investigation, we report that multiple H₂O₂ treatments induce a chilling tolerance comparable to cold acclimation in mung bean seedlings. However, in their response to light, the mechanisms of H₂O₂- and cold-induced acclimation could be distinguished. Participation of GSH and (Ca²⁺)ₓₜ in the H₂O₂-triggered tolerance in mung bean seedlings was also investigated in this study.

Materials and Methods

Plant materials and growth conditions. Seeds of Vigna radiata cv. Tainan No. 5 (TN5), a chilling-sensitive cultivar, were purchased from the local (Pu-Tze Township) farmers’ association. Seeds were germinated in pots containing a 1:1:1 perlite : 1 vermiculite : 1 peat (by volume) mixture. Seeded pots were placed in a greenhouse at 25 °C for 7 d with a 14 h light/10 h dark regime. Plants were watered daily without adding any nutrient.

Treatments. For cold acclimation, the 5-d-old seedlings were chilled at 10 °C for 48 h in the light or dark separately. Based on the survival curve of 4 °C-chilled mung bean seedlings (Yu et al., 2002), 36 h was chosen as the treatment time for 4 °C-chilling treatments in either light or dark. In addition, mung bean seedlings were chilled at 4 °C for different time periods to explore the acclimation and de-acclimation of H₂O₂-induced chilling tolerance. The photon flux density of all light period was 100 μmol·m⁻²·s⁻¹ supplied by cool white fluorescent lamps (FL40BR; China Electric Apparatus Ltd., Shanghai, China). A normal growth temperature (25 °C) was used as the experimental control in each experiment. For H₂O₂ pretreatment, the 7-d-old seedlings were sprayed to the runoff point with 200 mM H₂O₂ for from zero to five times before receiving a 4 °C chilling in light for 0–5 d. To test whether the H₂O₂-induced chilling tolerance could persist at room temperature, seedlings treated twice with H₂O₂ were incubated at 25 °C for 0, 12, 24, 36, 48, or 60 h before chilling exposure. For calcium treatment, the surfaces of the leaves of 7-d-old seedlings were also sprayed to the runoff point with 10 mM CaCl₂ containing 0.02% (v/v) Triton X-100. At the same time, a 0 mM control [i.e., water containing 0.02% (v/v) Triton X-100] was also conducted. Note that the residual CaCl₂ on the seedlings leaves would influence the accuracy of measurements of electrolyte leakage determined after the chilling treatment. Therefore, before a chilling treatment, the CaCl₂-treated plants were rinsed thoroughly with distilled water to remove the residual Ca²⁺ on leaves. Any two consecutive applications of H₂O₂ or CaCl₂ were separated by a 3-h interval unless otherwise noted. After treatment, the treated plants were then immediately transferred to a 4 °C growth chamber for chilling treatment as described above.

To inhibit GSH synthesis, the pots of 5-d-old seedlings were soaked individually in a tray containing ≈1 cm depth of 1 mM BSO aqueous solution (Kocsy et al., 2000b; Yu et al., 2002). The plants were cultivated in BSO-containing water for 2 d before receiving a chilling treatment. Mung bean seedlings were also pretreated with 1 mM BSO before receiving water, H₂O₂, or a 10 °C acclimation pretreatment, followed by a chilling treatment (4 °C, 36 h) in the light or dark.

To prove that the calcium source does in fact induce the chilling tolerance, potted seedlings were treated with either 10 mM EGTA, an extracellular calcium chelator, or 100 μM ruthenium red, an intracellular calcium blocker, for 12 h. Otherwise, conditions were identical to BSO treatment. Meanwhile, control plants were soaked in water alone. The effect of treatments on chilling tolerance was evaluated by measuring electrolyte leakage after a chilling treatment.

GSH analysis. To study the extent of GSH involvement in the increased tolerance produced by repeated H₂O₂ treatments, fluctuation of cellular GSH levels in seedlings was monitored after the stress at 4 °C for different periods (0, 12, 24, 36, 48, or 60 h) either in the light or in the dark.

GSH extractions followed the procedure of Anderson et al. (1992). Mung bean leaves (1 g fresh weight) were homogenized in 3 mL of ice-cold acidic extraction buffer (6% (w/v) metaphosphoric acid (pH 2.8), containing 1 mM EDTA), using a Polytron homogenizer (model PT-3100; Kinematica AG, Littau, Switzerland) at top speed for 1 min. Homogenates were centrifuged at 25,000×g for 20 min at 4 °C. The resulting supernatants were mixed with 60 mg of polyvinylpyrrolidone (PVPP) (Adams and Liyanage, 1991), and the slurry was centrifuged at 13,600×g for 20 min at room temperature. Supernatants were immediately analyzed for total glutathione contents. Total GSH content of mung bean seedlings was determined according to the procedure of Adams and Liyanage (1991) with minor modifications. The rate of absorption changes at 412 nm was monitored in a dual-beam spectrophotometer (model U-2001; Hitachi, Tokyo). The amount of GSH in the extracts was determined by comparing the rates of absorption change at 412 nm to those produced by standard GSH samples under the same reaction conditions.
**Electrolyte Leakage.** Leaf discs, 1 cm in diameter, from primary leaves were promptly collected at the end of each experiment. Three leaf discs were immersed in a 25-mL plastic vial containing 10 mL of double-distilled water. Electrolyte leakage was measured according to the procedure of Palate et al. (1977) using a SC-17 conductivity meter (model SC17A; Suntex Instrument, Taipei, Taiwan). The leaf disc suspension was shaken for 2 h before the first conductivity reading was taken. The vial was frozen overnight at −70 °C and then removed from the freezer and shaken until it reached ambient temperature, and conductivity was measured again. Leakage is described by the initial conductivity, expressed as a percent of the conductivity after the freezing and thawing.

**Protein Determination.** Protein was determined by the method of Bradford (1976) using lyophilized BSA (Bio-Rad, Hercules, CA) as a standard.

**Results**

Mung bean seedlings sprayed twice with 200 mM H$_2$O$_2$ showed reductions in electrolyte leakage after they had been chilled at 4 °C for 36 h under light irradiation (100 μmol m$^{-2}$ s$^{-1}$) (Fig. 1). The data indicated that a 3-h interval separating the two H$_2$O$_2$ applications induced the highest tolerance (Fig. 1). Notably, a 3-h interval gave electrolyte leakage of 21% ± 2%, which was even lower than that produced by 10 °C acclimation (29% ± 1%) (Fig. 1). The control and one-time H$_2$O$_2$-treated seedlings both showed relatively higher electrolyte leakages of 72% ± 4% and 50% ± 3%, respectively (Fig. 1). Two or three applications gave the greatest tolerance to 4 °C chilling periods of 1 or 2 d (Fig. 2). Two applications, but not three, gave significant protection to 3 d of 4 °C chilling (Fig. 2). However, after 4 d of 4 °C chilling, the electrolyte leakage of seedlings receiving repeated H$_2$O$_2$ treatments returned to 71% ± 8%, about the same level of controls (Fig. 2). For up to 5 d of chilling, plants acclimated at 10 °C maintained a relatively lower electrolyte leakage in comparison with H$_2$O$_2$-treated plants (Fig. 2).

We have demonstrated that high levels of GSH are important prerequisites for H$_2$O$_2$-induced chilling tolerance in plants.
Seedlings receiving two H\textsubscript{2}O\textsubscript{2} treatments had higher GSH levels than did 10 °C acclimated, single H\textsubscript{2}O\textsubscript{2}-treated, and water-treated control seedlings (Fig. 4A), even without chilling treatments. Meanwhile, 10 °C acclimated seedlings accumulated only a low GSH level (45% ± 5% nmol per mg of protein), similar to that of the water-treated control (43% ± 24% nmol per mg of protein) without chilling (Fig. 4A). When 10 °C acclimated seedlings were chilled at 4 °C in the light, small increases in their GSH levels for chilling periods over 24 h were observed (Fig. 4A). The GSH levels in seedlings acclimated at 10 °C and then exposed to 4 °C in the dark increased in a time-dependent way and peaked after 48 h. In either light or dark, seedlings challenged with H\textsubscript{2}O\textsubscript{2} either once or twice maintained considerably higher GSH amounts than that of water control throughout the period of chilling stress (Fig. 4B). The double H\textsubscript{2}O\textsubscript{2} treatment showed the most prominent effect on GSH accumulation, and the high GSH level persisted during entire chilling period (Fig. 4).

Using BSO to inhibit glutathione synthesis shows the degree to which glutathione participates in the induction of chilling tolerance in mung beans (Table 1). Addition of BSO effectively reduced the GSH of seedlings to very low levels compared with those in untreated plants (Table 1). BSO blocked, at least in part, the effect of H\textsubscript{2}O\textsubscript{2} on the electrolyte leakage of seedlings both in the light and the dark (Table 1). Interestingly, BSO had no influence on electrolyte leakage by 10 °C acclimated seedlings in the light; however, electrolyte leakage was increased in the dark (Table 1).

Stress signals triggered by various stimuli are mediated through (Ca\textsuperscript{2+})\textsubscript{cyt} to activate proper responses in plants (Knight, 2000; Knight et al., 1996; Sanders et al., 1999). We therefore propose that (Ca\textsuperscript{2+})\textsubscript{cyt} mediates the H\textsubscript{2}O\textsubscript{2}-triggered signal transduction, leading to chilling tolerance in mung bean seedlings. To test this possibility, the Ca\textsuperscript{2+} fluxes originating from extracellular or intracellular Ca\textsuperscript{2+} pools were disturbed by EGTA or ruthenium red, respectively (Table 2). Ruthenium red, EGTA, or a combination of both had no significant influence on GSH levels compared with untreated control (Table 2). Unexpectedly, block of calcium release from intracellular stores by ruthenium red slightly lowered the electrolyte leakage of 10 °C acclimated, singly and doubly H\textsubscript{2}O\textsubscript{2}-treated plants (Table 2). However, the electrolyte leakages of these cold-acclimated and H\textsubscript{2}O\textsubscript{2}-treated plants were remarkably increased by chelating extracellular Ca\textsuperscript{2+} by EGTA. The combination of EGTA with ruthenium red gave the electrolyte leakage close to that of EGTA alone (Table 2).

If Ca\textsuperscript{2+} influx from extracellular pools is a link between H\textsubscript{2}O\textsubscript{2} stimulation and the tolerance response, then the protective effect of H\textsubscript{2}O\textsubscript{2} ought to be substituted by exogenous application of Ca\textsuperscript{2+}. As expected, mung bean seedlings treated once with 10 mM CaCl\textsubscript{2} showed an electrolyte leakage of 35% ± 4%, approaching that of seedling receiving a single H\textsubscript{2}O\textsubscript{2} treatment (33% ± 4%) (Table 3). In addition, a repeated CaCl\textsubscript{2} treatment also lowered the electrolyte leakage of seedlings to the same level as a repeated H\textsubscript{2}O\textsubscript{2} treatment (Table 3). These data also imply that the H\textsubscript{2}O\textsubscript{2}-induced signaling pathway

**Table 1. Effect of buthionine sulfoximine (BSO) on glutathione level and electrolyte leakage of mung bean seedlings in the light or dark; values are means ± s. (n = 3).**

| Treatment | Glutathione (nmol per mg of protein) | Electrolyte leakage (%) |
|-----------|----------------------------------|-------------------------|
| In the light | H\textsubscript{2}O | BSO | H\textsubscript{2}O | BSO |
| H\textsubscript{2}O | 96.0 ± 13.4 | ND | 82.6 ± 4.6 | 78.5 ± 10.0 |
| Ac | 24.4 ± 4.5 | ND | 16.5 ± 1.3 | 19.2 ± 1.0 |
| 1× H\textsubscript{2}O\textsubscript{2} | 158.5 ± 43.7 | ND | 49.7 ± 8.4 | 65.9 ± 1.2 |
| 2× H\textsubscript{2}O\textsubscript{2} | 302.2 ± 65.5 | ND | 20.3 ± 3.0 | 45.7 ± 0.3 |
| In the dark | H\textsubscript{2}O | BSO | H\textsubscript{2}O | BSO |
| H\textsubscript{2}O | 135.2 ± 8.4 | ND | 76.1 ± 8.9 | 72.4 ± 10.0 |
| Ac | 262.8 ± 59.7 | 7.0 ± 27.8 | 20.2 ± 4.8 | 51.0 ± 7.8 |
| 1× H\textsubscript{2}O\textsubscript{2} | 214.0 ± 14.7 | 22.1 ± 16.4 | 51.5 ± 9.2 | 83.0 ± 2.5 |
| 2× H\textsubscript{2}O\textsubscript{2} | 317.2 ± 48.5 | ND | 16.4 ± 1.9 | 52.0 ± 4.8 |

\textsuperscript{Mung bean seedlings were pretreated with H\textsubscript{2}O\textsubscript{2} or 1 mm BSO in combination with H\textsubscript{2}O\textsubscript{2} 10 °C, 48-h acclimation (Ac), or H\textsubscript{2}O\textsubscript{2} [single (1×) or repeated (2×)]. After pretreatments, seedlings were immediately chilled at 4 °C for 36 h. All of these treatments were conducted in the light (100 μmol·m\textsuperscript{2}·s\textsuperscript{−1}) or in the dark, and then the glutathione level and electrolyte leakage of seedlings were measured.}

\textsuperscript{ND = not detected.}
leading to chilling tolerance is mediated through Ca²⁺. Interestingly, pretreatment with CaCl₂ followed by a H₂O₂ challenge (with 3 h between adjacent treatments) resulted in the electrolyte leakage of 28% ± 5%, which is higher than the value seen with seedlings subjected to repeated CaCl₂ or H₂O₂ treatment (Table 3). When the sequence of treatments was reversed, the seedlings pretreated with H₂O₂ before CaCl₂ showed electrolyte leakage of 17% ± 1%, about the same as that with repeated CaCl₂ and H₂O₂ treatments (Table 3).

Taken together, these observations suggest that the (Ca²⁺)cyt signals in the cells of mung bean seedlings may be influenced by H₂O₂ or CaCl₂ treatment. The CaCl₂ treatments induced only a relatively mild increase in GSH levels compared with the double H₂O₂ treatment (Table 3). This suggests that chilling tolerance triggered by CaCl₂ can be transmitted via a route unrelated to GSH accumulation.

**Discussion**

An earlier hypothesis about low-temperature acclimation in plants proposed that elevation of H₂O₂ in cells constituted an early signal leading to the physiological response, usually by activation of antioxidant mechanisms or modification of gene expression (Veal et al., 2007). This hypothesis was supported by the observation that exogenous application of H₂O₂ effectively increases chilling tolerance in plants (Prasad et al., 1994; Yu et al., 2002, 2003). The experiments reported herein show that mung bean seedlings pretreated with H₂O₂ repetitively develop a significantly higher chilling tolerance than do seedlings receiving only a single H₂O₂ treatment. The tolerance of repetitive treatment is even comparable to that of 10 °C acclimation (Fig. 1). The increased effect of a second application, together with the observation that there is an optimum separation time between the two treatments, is reminiscent of the “immune response” of animals. However, unlike the animal immune response, repeating the H₂O₂ treatment more than three times caused an adverse effect in chilling tolerance (Fig. 2).

Low-temperature acclimation is an inducible process to increase the low-temperature tolerance of plants, and its effects are transient (Sung et al., 2003; Wanner and Junitila, 1999). Although repeated H₂O₂ treatments induced the same degree of tolerance as did 10 °C acclimation, the tolerances induced by H₂O₂ and 10 °C acclimation were not intrinsically identical. The 10 °C acclimation-induced tolerance could persist for over 3 d at 4 °C; however, doubly H₂O₂-treated plants showed a significant decrease in their chilling tolerance when chilled at 4 °C for more than 2 d (Fig. 2). Thus, the double H₂O₂ treatment activated an acclimation mechanism somewhat different from that induced by 10 °C acclimation. On the other hand, plants made chilling-tolerant by 10 °C acclimation and double H₂O₂ treatment showed the same de-acclimation patterns at 25 °C (Fig. 3).

Among cellular components, GSH is a key component scavenging the chilling-induced ROS, thus enhancing the chilling tolerance of plants (Walker and McKersie, 1993). It has been shown that using BSO to inhibit GSH synthesis reversed the tolerance induced by chilling acclimation (Kocsy et al., 2000b; Yu et al., 2002, 2003). As predicted, a double H₂O₂ treatment induced a significant level of GSH in mung bean seedings in the light and in the dark (Fig. 4). Application of BSO to inhibit the GSH accumulation reduced the chilling tolerance of doubly H₂O₂-treated plants and increased the electrolyte leakage of mung bean seedlings (Table 1). Thus GSH plays a role in the chilling tolerance induced by H₂O₂. Karpinski et al. (1999) had observed a reduction in photo-oxidative damage after the treatment of arabidopsis leaves with H₂O₂. It was also reported that the amounts of GSH, glutamate, and glycine increased in maize grown at 5 °C; however, these changes were only significant in the light, not in the dark (Szalai et al., 1997). This clue stimulated us to study the influence of illumination on the low-temperature acclimation response in mung bean (Fig. 4). In 10 °C acclimated

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**Table 2. Effect of ruthenium red and ethyleneglycol-bis(amineoethylether)-N,N’-tetraacetatic acid (EGTA) on glutathione level and electrolyte leakage of mung bean seedlings; values are means ± sd (n = 3 to 6).**

| Treatment | H₂O | Ruthenium red | EGTA | Ruthenium red + | H₂O | Ruthenium red | EGTA | Ruthenium red + | H₂O | Ruthenium red | EGTA |
|-----------|-----|---------------|------|-----------------|-----|---------------|------|-----------------|-----|---------------|------|
| H₂O       | 99.7 ± 14.7 | 36.1 ± 9.9 | 174.0 ± 13.0 | 130.3 ± 26.9 | 82.7 ± 3.3 | 89.3 ± 0.5 | 83.2 ± 6.4 | 88.5 ± 1.4 |
| Ac        | 52.7 ± 26.4 | 64.5 ± 23.4 | 122.3 ± 23.6 | 87.9 ± 16.2 | 25.1 ± 2.5 | 19.3 ± 1.4 | 51.4 ± 0.6 | 46.1 ± 1.1 |
| 1× H₂O₂   | 208.3 ± 23.3 | 217.9 ± 7.6 | 271.0 ± 9.3 | 216.9 ± 5.5 | 53.9 ± 4.3 | 52.1 ± 4.0 | 62.4 ± 5.7 | 64.4 ± 6.7 |
| 2× H₂O₂   | 370.3 ± 41.5 | 352.7 ± 11.6 | 410.2 ± 23.7 | 377.4 ± 43.2 | 29.1 ± 3.4 | 25.9 ± 2.5 | 62.3 ± 0.9 | 53.1 ± 1.9 |

**Table 3. Effects of calcium on glutathione levels and electrolyte leakage in response to chilling in mung bean seedlings; values are means ± sd (n = 3).**

| Treatment | Glutathione (nmol per mg of protein) | Electrolyte leakage (%) |
|-----------|-----------------------------------|-------------------------|
| H₂O       | 122.8 ± 6.1                       | 68.0 ± 7.6              |
| Ac        | 41.5 ± 18.4                       | 20.4 ± 1.9              |
| H₂O₂      | 178.0 ± 41.0                      | 33.4 ± 4.3              |
| H₂O₂/H₂O₂ | 443.9 ± 73.7                      | 17.1 ± 1.9              |
| CaCl₂     | 143.1 ± 14.3                      | 35.2 ± 4.1              |
| CaCl₂/CaCl₂ | 225.2 ± 36.6                     | 19.5 ± 1.6              |
| CaCl₂/H₂O₂ | 214.6 ± 66.8                     | 28.3 ± 5.0              |
| H₂O₂/CaCl₂ | 179.7 ± 43.0                     | 17.0 ± 1.3              |

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- **Yu et al., 2002, 2003.** The experiments reported herein show that mung bean seedlings pretreated with H₂O₂ repetitively developed a significantly higher chilling tolerance than do seedlings receiving only a single H₂O₂ treatment. The tolerance of repetitive treatment is even comparable to that of 10 °C acclimation (Fig. 1). The increased effect of a second application, together with the observation that there is an optimum separation time between the two treatments, is reminiscent of the “immune response” of animals. However, unlike the animal immune response, repeating the H₂O₂ treatment more than three times caused an adverse effect in chilling tolerance (Fig. 2).

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seedlings stressed at 4 °C in the dark, GSH gradually accumulated to a very high level and reached a peak value after 48 h of chilling (Fig. 4B). However, synthesis of GSH was inhibited in the light (Fig. 4A). This observation is different from the results of Karpinski et al. (1999). Furthermore, in the light, BSO-diminished GSH accumulation had little influence on the chilling tolerance of plants (Table 1). These findings suggest that, upon illumination, the 10 °C acclimation treatment triggered a pathway, independent of GSH accumulation, to protect seedlings from chilling damage. The high GSH level in cells after 10 °C acclimation in the dark seems to be necessary for chilling acclimation (Table 1). Besides, with or without illumination, GSH was found to be essential for chilling tolerance induced by both single and repeated H2O2 treatments (Table 1). Therefore, illumination should be able to modify the signal transduction pathway leading to cold acclimation. On the other hand, H2O2-induced cold tolerance was mediated through light-independent pathway. Higher plants sense the photoperiodic changes by using three major classes of photoreceptors: red/far-red absorbing phytochromes, blue/ultraviolet A absorbing cryptochromes, and phototropins (Wang, 2005). Among them, phytochromes and cryptochromes were evidenced to involve in the control of cold-regulated gene expression of Hordeum vulgare L. (Crosatti et al., 1999). Therefore, the perception of illumination by some photoreceptors may also participate in the regulation of GSH synthesis and, perhaps independently, in the adaptation of mung bean seedlings to chilling. Nevertheless, the negative regulation of GSH levels by light irradiation warrants further investigation.

Yang and Poovaiah (2002) indicated that a close interaction exists between intracellular H2O2 and (Ca2+)cyt in response to biotic and abiotic stresses. This study indicated that an increase in (Ca2+)cyt boosted the generation of H2O2 (Yang and Poovaiah, 2002). Consistent with this result, EGTA moderately inhibited the development of chilling tolerance in 10 °C acclimated and H2O2-treated plants, as reflected by increased electrolyte leakage after seedlings were chilled at 4 °C for 36 h (Table 2). Disruption of the Ca2+ flux from intracellular pools by ruthenium red had no significant effect on the chilling tolerance induced either by 10 °C acclimation or H2O2 treatment (Table 2). Thus the Ca2+ signal originating from the ruthenium red sensitive intracellular Ca2+ pools appears to be unrelated to the process for developing chilling tolerance in mung bean seedlings. In contrast, 10 °C acclimation and H2O2 pretreatments acclimatize plants to chilling through calcium signals originating from extracellular Ca2+ pools. Interestingly, the EGTA and ruthenium red had little effect on GSH levels in this study (Table 2). This observation differs from the report that Ca2+ induced GSH accumulation and stress tolerance in Oryza sativa L. (Lu et al., 1999). Thus, Ca2+ may also induce another protective mechanism besides GSH.

In addition to serving as a link in a signaling cascade, fluctuation of (Ca2+)cyt could be a mechanism that leads plants to remember what they have suffered (Knight et al., 1996). This inference comes from the observation that arabinodioses treated with either sublethal cold or H2O2 modifies its Ca2+ signature in response to subsequent cold stress as compared with an untreated control (Knight et al., 1996). Indeed, in Table 3, 10 mM CaCl2 induced a chilling tolerance approaching that of H2O2 treatment. In addition, CaCl2 could replace each of two independent H2O2 treatments without a substantial influence on induction of chilling tolerance (Table 3). This evidence suggests that Ca2+ is one of the downstream messages involved in H2O2-triggered signal transduction.

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