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Modulation of alternative oxidase to enhance tolerance against cold stress of chickpea by chemical treatments

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ABSTRACT

The alternative oxidase (AOX) is the enzyme responsible for the alternative respiratory pathway. This experiment was conducted to examine the influence on cold tolerance ability of chickpea (*Cicer aurentium* cv. Müfitbey) seedlings of AOX activator (pyruvate), AOX inhibitor (salicylhydroxamic acid (SHAM)) and an inhibitor of the cytochrome pathway of respiration (antimycin A) treatments. 5 mM pyruvate, 2 μM antimycin A and 4 mM SHAM solutions were exogenously applied to thirteen-day-old chickpea leaves and then the seedlings were transferred to a different plant growth chamber arranged to 10/5 °C (day/night) for 48 h. Cold stress markedly increased the activities of antioxidant enzymes compared to controls. Pyruvate and antimycin A significantly increased the cold-induced increase in antioxidant activity but SHAM decreased it. Cold-induced increases in superoxide anion, hydrogen peroxide, and lipid peroxidation levels were significantly reduced by pyruvate and antimycin A, but increased by SHAM treatment. Pyruvate and antimycin A application increased both the activity and protein expression of AOX in comparison to cold stress alone. However, SHAM significantly decreased activity of AOX but did not affect its expression. Total cellular respiration values (TCRV) supported the changes in activity and expression of AOX. While TCRV were increased by cold and pyruvate, they were significantly reduced by SHAM and especially antimycin A. These results indicate that pyruvate and antimycin A applications were effective in reducing oxidative stress by activating the alternative respiratory pathway as well as antioxidant activity. Furthermore, direct activation of AOX, rather than inhibition of the cytochrome pathway, was the most effective way to mitigate cold stress.

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Introduction

One of the most severe abiotic stressors, cold stress leads to serious dysfunction at the cellular level, including protein denaturation, damage to membranes and genetic material, excessive production of reactive oxygen species (ROS), and deterioration in cellular redox status (Wang et al., 2003; Pearce, 1999). When plants are exposed to cold stress, ROS production excessively increases due to the restriction of CO_2 fixation in chloroplasts and mitochondrial electron transport chain (Davidson and Schiestl, 2001; Suzuki and Mittler, 2006). Therefore, to cope with ROS-induced oxidative damage, plants have developed complex defense mechanisms, including enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT), glutathione reductase (GR) and non-enzymatic antioxidants such as ascorbic acid and glutathione (Kang and Saltveit, 2002; Erdal, 2012).

The alternative oxidase (AOX) is the enzyme responsible for the alternative respiratory pathway. It has been reported that activity of AOX also increases under stress conditions as do activities of antioxidant enzymes (Vanlerberghe and McIntosh, 1992a; Purvis and Shewfelt, 1993). This enzyme, found at low concentrations under unstressed conditions, was previously considered to act in only heat production in thermogenic plants because free energy arising from electron flow is released as heat while the electrons passing thorough AOX reduce O_2 to H_2O (Meeuse, 1975; Vanlerberghe and McIntosh, 1997). Later, it was determined that this enzyme had

Abbreviations: AOX, alternative oxidase; APX, ascorbate peroxidase; BSA, bovine serum albumin; CAT, catalase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetra acetic acid; ETC, electron transport chain; GPX, guaiacol peroxidase; GR, glutathione reductase; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitro blue tetrazolium; $O_2^{\bullet-}$, superoxide anion; ROS, reactive oxygen species; SHAM, salcylhhidroxamic acid; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCRV, total cellular respiration values.

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critical roles in many metabolic processes as well as internal heat production (Purvis, 1997; Wagner and Moore, 1997).

AOX is encoded in a multigene family that includes the *Aox1* and *Aox2* subfamilies. These genes may vary according to plant species. The *Aox1* subfamily exists in both monocotyledon and eudicotyledon plant species and is induced with environmental stress conditions. On the other hand, the A*ox2* subfamily is present only in eudicotyledons and is expressed based on the development of plants (Considine et al., 2002; Juszczuk and Rychter, 2003; Polidorosa et al., 2009).

AOX helps to protect the intracellular oxygen–water concentration with the formation of an H_2O molecule for four electrons passing through AOX, and prevents the blockade of electron flow. Thus, AOX has a limiting effect on ROS production (Maxwell et al., 1999; Møller and Kristensen, 2004). On the other hand, AOX helps in maintaining the integration among respiration and other metabolic processes by contributing to dissipation of blockage among carbon metabolism, electron transport and the ATP cycle (Finnegan et al., 2004; Vanlerberghe et al., 2009).

AOX is located as a homodimer in the inner membrane of mitochondria (Umbach and Siedow, 1993; Siedow et al., 1995). These dimer bounded covalent bonds are inactive forms of AOX (Vanlerberghe et al., 1999). They are activated with the reduction of the covalent bonds (Umbach and Siedow, 1993). The AOX enzyme can be activated or inactivated by various molecules. The active monomer units of AOX are further activated with α-keto acids such as pyruvate (Millar et al., 1993; Pastore et al., 2001; Gelhaye et al., 2004). This activation arises from the allosteric impact of pyruvate (Day et al., 1994; Vanlerberghe et al., 1995). In addition, antimycin A indirectly increases the AOX activity by inhibiting the cytochrome pathway of respiration (Vanlerberghe and McIntosh, 1992b). By contrast, salicylhydroxamic acid (SHAM) gives rise to the inhibition of AOX (Schonbaum et al., 1971; Michae, 1989).

Despite many reports suggesting the importance of alternative respiration on cold stress tolerance in plants, there are a limited number of studies examining the ability of plants to tolerate cold stress by activating or inactivating this pathway with exogenous applications. Recently, it has been reported that exogenous pretreatment of hydrogen peroxide (H_2O_2), which has been implicated as a diffusible signal molecule in addition to its endogenous oxidant function, gained resistance against cold stress in wheat seedlings by increasing the activity of alternative respiration pathways, whereas SHAM application decreased the resistance gained with H_2O_2 pretreatment (Feng et al., 2008).

In the present study, we aimed to elucidate the influence of alternative respiration on the cold resistance of chickpea seedlings pre-treated with AOX activator (pyruvate), AOX inhibitor (salicylhydroxamic acid (SHAM)), or an inhibitor of the cytochrome pathway of respiration (antimycin A).

Materials and methods

Plant material, growth conditions, and applications

After the registration, chickpea (*Cicer arietinum* cv. Yaşa-05) seeds were sterilized for 10 minutes with 10% NaOCl solution, and the seeds were washed a few times with distilled water. Chickpea seeds were grown for 13 days in sand culture and in a plant growth chamber at a constant temperature regime of 25 ± 1 °C (day/night) for a 16 h photoperiod at 40 ± 5% relative humidity. Optimum concentrations of pyruvate antimycin A and salicylhydroxamic acid (SHAM) were determined with preliminary works by looking to changes at the malondialdehyde (MDA) level and then on the 13th day of growth, 5 mM pyruvate 2 μM antimycin a and 4 mM SHAM were exogenously applied to the leaves of chickpea seedlings. After 12 h from the applications, seedlings were transferred to a different plant growth chamber set to 10/5 °C (day/night). The control group's growth was continued on cycle of 25 ± 1 °C (day/night). After 48 h from the low temperatures applications, leaves were harvested for biochemical analyses.

Enzyme extraction and assays

Homogenized leaf tissues (0.5 g) were ground in a mortar with liquid nitrogen and extracted in 500 μL of 10 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinyl pyrrolidone and 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. The supernatant was used as an enzymes source (SOD, GPX, CAT, GR and APX). Leaf soluble protein content was determined according to the method of Bradford (1976) by using bovine serum albumin as standard.

SOD activity was assayed according to the method of Agarwal and Pandey (2004). One unit of SOD activity was defined as the amount of the enzyme required to cause a 50% inhibition of nitro blue tetrazolium (NBT) reduction rate by monitoring the absorbance at 560 nm.

For the measurement of GPX activity, the method of Yee et al. (2002) was used. The method is based on the decomposition rate of H_2O_2 by peroxidase, with guaiacol as a hydrogen donor. The amount of enzyme that caused an increase of 0.01 absorbance was defined as one unit of GPX activity and expressed as U mg^{-1} protein.

The CAT activity was determined by evaluating the rate of decline in absorbance at 240 nm of a solution of 12.5 mM H_2O_2 in 50 mM KH_2PO_4 (pH 7.0) at 30 °C. The amount of enzyme catalyzing the decay of 1 μmol H_2O_2 per minute calculated from the extinction coefficient for H_2O_2 at 240 nm of 0.036 cm^2 μmol^{-1} and is expressed as one unit of the CAT enzyme activity (Gong et al., 2001).

The APX activity was assayed according to Nakano and Asada (1981) by listing the ascorbate oxidation rate at 290 nm (ε = 2.8 mM^{-1} cm^{-1}).

GR activity was based on the method of Foyer and Halliwell (1976). The oxidized glutathione (GSSG)-dependent oxidation of NADPH was followed by a decrease in the absorbance at 340 nm. One unit of GR activity was defined as 1 mmol ml^{-1} GSSG reduced min^{-1}.

Measurement of superoxide anion production, hydrogen peroxide level and malondialdehyde content

The assay of superoxide ($O_2^{\bullet -}$) production was based on the method of Elstner and Heupel (1976). The production rate of $O_2^{\bullet -}$ was calculated by using sodium nitrite as a standard solution.

Hydrogen peroxide (H_2O_2) content was measured as described by Velikova et al. (2000). The content of H_2O_2 was calculated by using the standard curve.

To determine the MDA content, a thiobarbituric acid (TBA) test was used as described by Heath and Packer (1968). The amount of MDA-TBA complex was determined from the extinction coefficient 155 $(mmol\ L^{-1})^{-1}$ cm^{-1}.

Isolation of mitochondria of chickpea

5 g tissue from the fresh leaves was homogenized in 20 ml of extraction buffer containing 0.4 M mannitol, 50 mM MOPS (pH 7.2), 2 mM ethylene glycol tetra acetic acid (EGTA), 4 mM L-cysteine, 20 mM β-mercaptoethanol, 0.6% (w/v) polyvinylpyrrolidone (PVP) and 0.5% (w/v), bovine serum albumin (BSA) at 4 °C. After the homogenate was centrifuged at 400 *g* for 5 min, supernatant was centrifuged again for 10 min at 2000 × *g*. The supernatant portion

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