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Activities and Isozyme Profiles of Antioxidant Enzymes in Intercellular Compartment of Overwintering Barley Leaves

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Overwintering plants should survive the various biotic and abiotic stresses that occur during winter. Previous studies indicated that active oxygen species are involved in freezing, dehydration, anoxia and pathogen infections. As the importance of the events that occur in the intercellular compartment became apparent in disease resistance, we examined the nature of intercellular antioxidant enzymes in order to access their possible involvement in the winter hardiness of barley. The levels of intercellular peroxidase, catalase, and SOD activities on the unit protein basis were 394, 18, and 9% of those of cellular activities, respectively. Major intercellular peroxidase isoforms consisted of four neutrals and four basic forms; whereas major cellular isoforms were two basic forms. Out of the two major catalase isoforms a higher molecular weight form was predominantly abundant in both cellular and intercellular compartments. Among the five major cellular SOD isoforms, three were also present in the intercellular compartment. The presence of substantial amounts of intercellular antioxidant enzymes in overwintering barley leaves may suggest the involvement of these enzymes in the tolerance mechanism to the various stresses that occur during winter.

Keywords: Barley, Catalase, Intercellular compartment, Peroxidase, Superoxide dismutase (SOD), Winter hardiness.

Introduction

Overwintering plants have a freezing tolerance for surviving subzero temperatures. Winter hardiness, however, is a complex trait that involves tolerance to dehydration, anoxia, disease, as well as freezing. Overwintering crops must tolerate the combinations and severity of these stresses that occur during winter.

Even though there are distinct differences among the winter stresses, oxidative stress may be a common element. Abiotic and biotic stresses such as freezing (Kendall and McKersie, 1989), chilling (Prasad *et. al.*, 1994), desiccation (Senaratna, 1987), and pathogen infection (Lamb and Dixon, 1997; Oh *et al.*, 1999) lead to an increased proliferation of toxic oxygen species. Therefore, various antioxidative enzymes have been shown to be involved in the resistance mechanisms to the oxidative stresses. Overexpression of superoxide dismutase (SOD) increases the winter survival of alfalfa (McKersie *et al.*, 1999). Activities of catalase and ascorbate peroxidase are lowest in chilling sensitive maize (Hodges *et al.*, 1997). SOD and catalase activities increase in barley leaves infected with pathogen (Vanacker *et al.*, 1998) and peroxidase activity increases by the elevated production of H_2O_2 in transgenic plants (Wu *et al.*, 1997).

At temperatures below 0°C ice typically forms in the intercellular spaces of plant tissues and the accumulation of ice can cause physical disruption in cells and tissues (Levitt, 1980). Ice formation in the intercellular space results in severe cellular dehydration that causes major freezing injury (Steponkus and Webb, 1992). Specific proteins accumulate in the intercellular space of barley, rye, wheat, kale and winter canola during cold acclimation (Antikainen and Griffith, 1997) and some of the proteins show antifreeze activity. Antifreeze proteins (AFPs) have the ability to modify the formation and growth of ice and inhibit ice recrystallization (Griffith *et al.*, 1997), thus contributing to the protection of the cells from freezing injury.

The intercellular space in plant tissue is a key compartment between the plasma membranes connecting cells to cells and the external environment. The importance of the series of events occurring in the intercellular space in plant-pathogen interactions has been documented (Mehdy, 1994; Lamb and Dixon, 1997). Wall-bound peroxidases produce superoxide

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radicals and SODs convert superoxide radicals to H_2O_2 and O_2. Hydrogen peroxide may have an immediate antimicrobial effect and act as a signal molecule to induce defense-related genes (Lamb and Dixon, 1997). Catalase and ascorbate peroxidases destroy hydrogen peroxide in order to control its concentration at sublethal levels. Considering oxidative stress as a fundamental element in freezing and pathogen infection, similar antioxidant enzyme systems are expected to be functioning in the intercellular space of freezing-tolerant plants. To test our hypothesis we examined intercellular antioxidant enzyme systems in overwintering barley leaves.

Materials and Methods

Chemicals and Plant Material All of the chemicals used in the experiment were purchased from Sigma (St. Louis, MO, USA). Plants of four Korean barley varieties (Jinkwangbori, Hinchalssalbori, Naehanssalbori and Olbori) were grown in the field of the experimental farm of Chonbuk National University, Chonju, Korea, in 1998 and 1999. Healthy leaves that were free of any disease or stress symptoms were then harvested in early February and used for protein and enzyme assays.

Protein Extraction and Electrophoresis Proteins in cellular and intercellular compartments were extracted separately from the leaf tissues. The extraction buffer contained 50 mM Tris-HCl, pH 8.0, 20 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride and 10 mM β-mercaptoethanol (β-ME). Intercellular proteins were first extracted by centrifugation of the buffer-infiltrated leaves at 1,000 g for 10 min (Hon *et al.*, 1994). This has proven to be an effective procedure with cellular contamination of less than 2%. After intercellular space protein extraction the leaf tissues were ground under liquid N_2, and one gram of leaf powder was homogenized with 3 ml of the extraction buffer and 40 mg of polyvinylpolypyrrrolidone in a chilled mortar and pestle. The homogenate was centrifuged at 12,000 *g* for 20 min and the resulting supernatant was used as intercellular protein source. Protein levels were determined by the method of Bradford (1976) with bovine serum albumin as a standard. Heat denatured proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels according to the method described by Laemmli (1970) and visualized using silver stain (Westermeier, 1993).

Enzyme Assays and Isozyme Analyses The cellular and intercellular protein extracts prepared for protein analyses were also used for peroxidase and SOD analyses. Protein extracts for catalase were prepared by the same procedure except the dithiotreitol (DTT) where was substituted for β-ME. Thirty ug proteins in the extracts were used for activity and isozyme analyses otherwise indicated. Catalase activity was assayed by determining the rate of change in the absorbance at 240 nm in a reaction mixture (1.5 ml) that consisted of 50 mM potassium phosphate, pH 6.9, 11.6 mM H_2O_2 and 10 mM DTT at 25°C (Beers and Sizer, 1952). Catalase isozyme bands were developed on a 8% polyacrylamide gel by incubating the gel successively in the substrate (3.27 mM H_2O_2) and developing (1% potassium ferricyanide and 1% ferric chloride) solutions (Woodbury *et al.*, 1971). In the loading buffer for catalase, 60 mM DTT was substituted for β-ME. Peroxidase activity was assayed in a reaction mixture that consisted of 50 mM potassium phosphate, pH 6.4, 0.3 mM guaiacol, 0.14 mM H_2O_2 as described by Chance and Maehly (1955). Peroxidase isozymes were detected on ultrathin-layer IEF gels according to Wendel (1987). SOD activity was determined by the xanthine oxidase-nitro blue tetrazolium (NBT) assay (Oberley and Spitz, 1984). The reaction mixture (1 ml) contained 50 mM potassium phosphate, pH 7.8, 0.1 mM xanthine, 0.056 mM NBT, 1 mM diethylenetriamine pentaacetic acid, and 1 unit catalase. SOD isozymes were analyzed after separating proteins on 10% polyacrylamide gels as described (Yun and Lee, 1994).

Results and Discussion

Intercellular Proteins Wintering barley leaves contained 26 mg protein/g FW on average. Only about 0.4 percent (104 μg/g FW) of the total protein was in the intercellular compartment (Fig 1A). Profiles of intercellular polypeptides were drastically different from those of cellular polypeptides. As shown in Fig. 1B, there were at least nine major intercellular specific polypeptides ranging in size from 7 to 34 kDa. They could be divided into three groups by their size: three in 32 to 34 kDa (group 1), four in 20 to 23 kDa (group 2) and two less than 10 kDa in size (group 3). A varietal difference was indicated in one and two higher molecular weight polypeptides in the group 1 and 2, respectively.

Accumulation of intercellular proteins has been observed in both monocotyledonous and dicotyledonous overwintering plant species, but with different protein profiles. Intercellular protein contents in winter rye and barley leaves increase up to 145 and 110 μg protein/g FW, respectively, after cold acclimation for 7 weeks at 5/2°C (Antikainen and Griffith, 1997). Molecular weight of the major intercellular proteins ranges from 35 to 8 kDa. Our results indicate that field-grown plants also contain similar amounts of intercellular proteins similar in size to those in plants cold-acclimated in a controlled environment. At least six to eight of the proteins accumulating in rye and barley leaves have antifreeze activity (Antikainen and Griffith, 1997). Thus, some of the polypeptides detected in the intercellular compartment may contribute to freezing tolerance by the inhibition of formation, growth and recrystallization of ice.

Intercellular Antioxidant Enzymes The levels of intercellular peroxidase activity per unit of protein were about 4 times higher as 6.78 μmole/mg protein/min than those of cellular activity. On the fresh weight basis, however, the levels of intercellular activity were only 14% of those in the cellular activity (Fig. 2A). As indicated in Fig. 2B, major intercellular peroxidase isoforms were different from the cellular isoforms. Major intercellular isoforms consisted of the four neutral and four basic forms. Numerous acidic isoforms were also present as minor isoforms. Contrary to the isoforms in the intercellular space, the two basic isoforms were the major

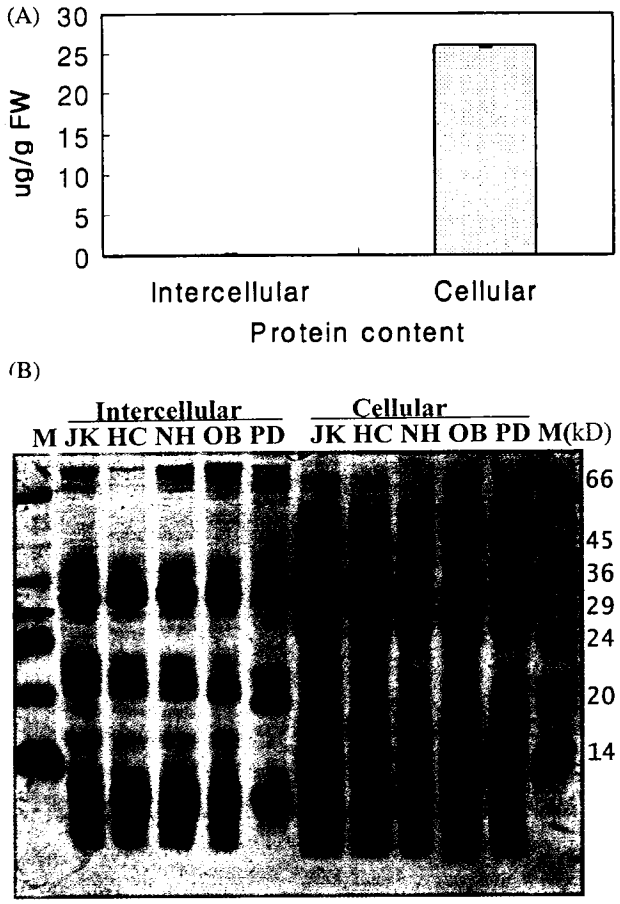

Fig. 1. Proteins in overwintering barley leaves. A. Cellular and intercellular protein contents. Values represent means±SE of the four varieties. B. Polypeptides in the cellular and intercellular compartments of the four barley varieties, Jinkwangbori (JK), Hinchalssalbori (HC), Naehanssalbori (NH) and Olbori (OB). A rye variety, Paldanghomil (PD) was used as a reference. Twenty micrograms of denatured proteins were separated by SDS-PAGE on a 12.5% polyacrylamide gel and detected by silver stain.

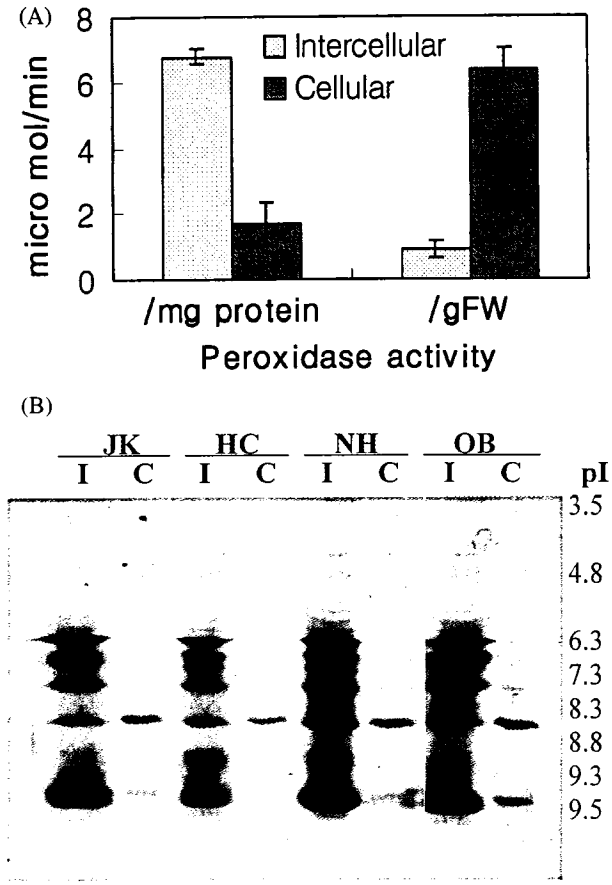

Fig. 2. Peroxidases in overwintering barley leaves. A. Cellular and intercellular peroxidase activities by the unit fresh weight or protein. Values represent means±SE of the four varieties. B. Peroxidase isoforms in the cellular (C) and intercellular (I) compartments of the four barley varieties, Jinkwangbori (JK), Hinchalssalbori (HC), Naehanssalbori (NH) and Olbori (OB). One microgram of proteins were separated on a ultra-thin IEF gel and detected as described in Materials and Methods.

enzymes in the cellular space.

Plants contain abundant intercellular peroxidase isoforms that are postulated to play an integral role in the polymerization of cell wall components including lignin, suberin and extension (Gaspar *et al.*, 1982). Cell walls are considered to play an important role in protecting cells from freezing and pathogen infection. Therefore, structural reinforcement by biosynthesis and modification of cell walls may contribute to freezing tolerance.

It has been reported that up to 20% of the peroxidase activity is contained in the apoplastic washing solution of barley primary leaves (Li *et al.*, 1989). Also, at least six peroxidase isozymes are present in the intercelluar compartment of barley leaves (Scott-Craig, 1995).

Plasma membrane bound peroxidases are involved in the production of reactive oxygen species to provide H_2O_2. The reactive oxygen species and H_2O_2 can act as immediate antibiotic agents. Cell wall peroxidases are also induced by pathogen infection and considered to play an important role in cell wall lignification during the hypersensitive reaction (Mehdy, 1994). Peroxidases participate in a variety of plant defense mechanisms in which H_2O_2 is often supplied by an oxidative burst (Lamb and Dixon, 1997). Some of the intercellular peroxidases found in overwintering barley leaves may participate in cell wall lignification. Ascorbate peroxidases are involved in the detoxification of H_2O_2, which may also contribute to enhanced freeze tolerance (Asada, 1997). Oxidation of phenolic compounds, catalyzed by certain types of peroxidases, may be required for cell expansion. In plant-pathogen interactions, the result is determined by the temporal distribution of responses and dynamic changes in the concentration of active oxygen species in the cellular and intercellular compartment (Tiedemann, 1997). Therefore, multiple isozymes that are detected in the intercellular fluid of overwintering barley leaves may reflect the complex nature of

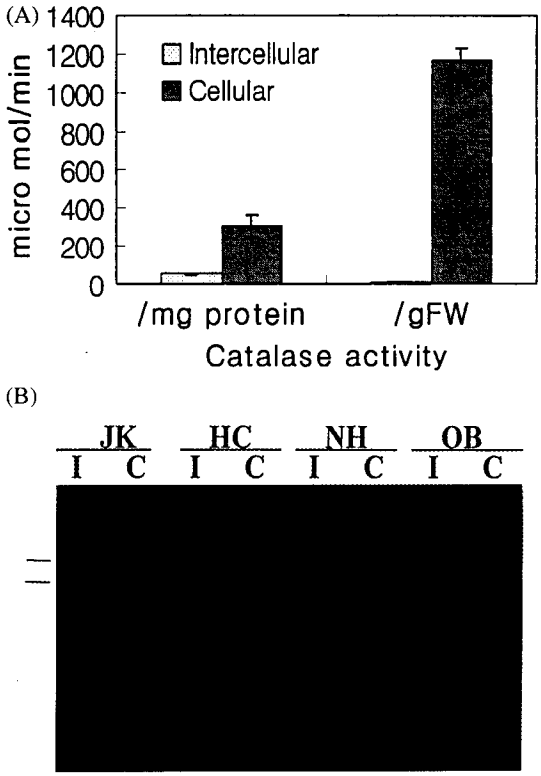

Fig. 3. Catalases in overwintering barley leaves. A. Cellular and intercellular catalase activities by the unit fresh weight or protein. Values represent means±SE of the four varieties. B. Peroxidase isoforms in the cellular (C) and intercellular (I) compartments of the four barley varieties, Jinkwangbori (JK), Hinchalssalbori (HC), Naehanssalbori (NH) and Olbori (OB). Thirty micrograms of proteins were separated by PAGE on a 8% polyacrylamide gel and detected as described in Materials and Methods.

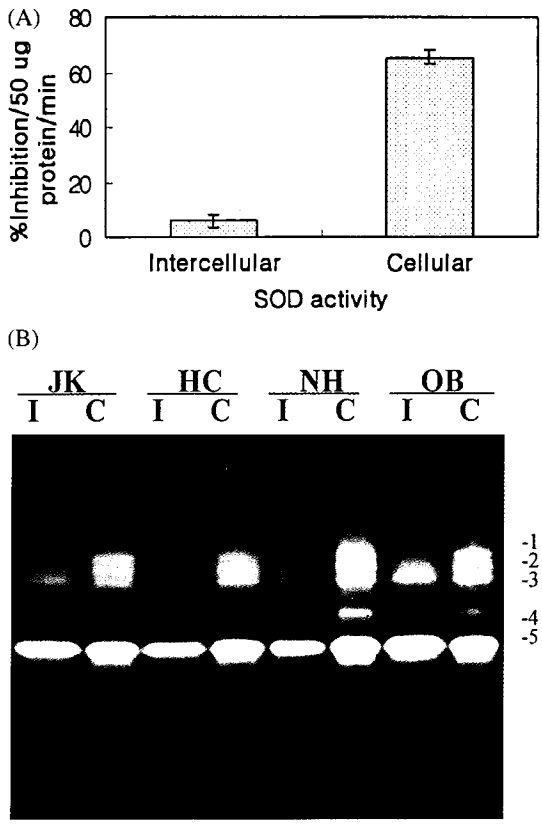

Fig. 4. Superoxide dismutases in overwintering barley leaves. A. Cellular and intercellular SOD activities by the unit protein. Values represent means±SE of the four varieties. B. SOD isoforms in the cellular (C) and intercellular (I) compartments of the four barley varieties, Jinkwangbori (JK), Hinchalssalbori (HC), Naehanssalbori (NH) and Olbori (OB). Thirty micrograms of proteins were separated by PAGE on a 10% polyacrylamide gel and detected as described in Materials and Methods.

events occurring in the compartment in which the peroxidases are located.

Levels of intercellular catalase activity were about 0.6 percent of the total leaf activity on the unit fresh weight basis (Fig. 3A). Intercelluar catalase activity on the unit protein basis, however, accounts for about 18% of the cellular activity. Two major catalase isoforms were found in the overwintering barley leaves. The isoform of higher molecular weight was present predominantly, but the isoform with lower molecular weight was much less in both locations (Fig. 3B).

Catalases detoxify hydrogen peroxide by converting it to water and oxygen molecules, and are mostly confined in peroxisomes. Two barley catalase genes, Cat1 and Cat2, have been isolated (Skadsen *et al.*, 1995) and subunits of 57 and 53 kDa are regulated differentially by light (Holtman *et al.*, 1998). There are few reports on intercellular catalase activity and it accounts for only about 0.25% of the total cellular activity in healthy leaves on a fresh weight basis. However, upon pathogen infection, the catalase activity increases up to 2.5% (Vanacker *et al.*, 1998). Our results indicated that intercellular catalase activity in overwintering leaves is higher than that found in leaves uninfected with pathogen, but lower than that found in infected leaves. Specific activity per unit protein, however, accounted for about 18% of the cellular activity. Considering the fact that catalases are the major enzyme destroying hydrogen peroxide, the higher specific activity and presence of the cellular isoforms in the intercellular compartment may indicate the possible involvement of catalases in the freezing tolerance by controlling the hydrogen peroxide concentration in the compartment.

SOD activity was also detected in the intercellular space and the levels were about 9 percent on the unit protein basis of those found in the cellular space (Fig. 4A). Levels of the intercellular SOD activity increase from about 1% to 3% of total leaf activity on fresh weight basis in the pathogen-infected leaves (Vanacker *et al.*, 1998). There were at least 5 major SOD isoforms, as shown in Fig. 4B. They were designated tentatively as SOD1, 2, 3, 4, and 5, respectively. The isoform SOD1 was most abundant in both intercellular

and cellular compartments, but SOD2 and 5 were not detected in the intercellular fluids. There were little varietal differences in the SOD isozyme profiles.

Active oxygen species are mainly generated in the chloroplasts and mitochondria during photosynthesis and respiration, respectively. Therefore, SODs located in the organelles and cytosol of plant cells have received attention for their roles in protecting subcellular components by catalyzing the dismutation of the superoxide radical to molecular O_2 and H_2O_2. Barley leaves contain the three different types of SODs (Fe-, Mn-, Cu/Zn-SOD) and their expression in young leaves is increased under oxidative stress (Casano *et al.*, 1994). Expression of the cytosolic Cu/Zn- and mitochondrial Mn-SODs increases during natural acclimation in both spring and winter wheat. Under freeze-thaw treatment expression of the mitochondrial SOD genes declines in spring wheat but remains unchanged in winter wheat. This suggests the involvement of SODs in the cold tolerance mechanism in wheat (Wu *et al.*, 1999). Overexpression of the mitochondrial SOD is associated with an increase in the winter survival of transgenic alfalfa (McKersie *et al.*, 1999). But, there are few reports on the intercellular SODs or their possible roles in the compartment under various oxidative stresses. Relatively higher levels of activity, and the presence of isoforms found in the intracellular compartment, may extend their protective roles to the outer layers of plasma membranes and other intercellular components in overwintering leaves.

Dual roles of reactive oxygen species require antioxidant enzyme systems for the control of the toxic oxygen under sublethal levels. The activities of the antioxidant enzymes found in the intercellular compartment were low relative to those found in total leaves. The volume of the intercellular fluid represents only 4.5% of the total cell volume of barley leaf (Winter *et al.*, 1993). However, the specific activities of intercellular peroxidase, catalase, and SOD on unit protein were 394, 18, and 9% of those of cellular activities. As the importance of antioxidant in the intercellular compartment becomes apparent in plant-pathogen interactions (Luwe, 1996), the presence of substantial amounts of intercellular catalase, peroxidase and SODs in overwintering barley leaves may suggest the involvement of these enzymes in the tolerance mechanism to the various stresses during winter. Temporal and dynamic regulations of reactive oxygen concentration may also be as important in the freezing tolerance as in the plant-pathogen interactions. Therefore, further investigations on the intercellular antioxidant enzymes will be needed in order to understand their roles under various stress conditions or developmental stages.

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