Salt Stress-Induced Dissociation from Cells of a Germin-Like Protein with Mn-SOD Activity and an Increase in its mRNA in a Moss, *Barbula unguiculata*

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To estimate the physiological roles of a germin-like protein (BuGLP) with Mn-SOD activity isolated newly from a moss, *Barbula unguiculata*, BuGLP mRNA levels during cell growth and the effects of methyl viologen and salt stress were studied. BuGLP mRNA levels were at their peak during the exponential phase of growth and decreased thereafter, but SOD activity was held at the same level as that during the exponential phase. When methyl viologen was present as a generator of superoxide the amount of BuGLP transcripts decreased, but that of SOD activity of BuGLP bound to the cell wall was not affected. The addition of NaCl to the cells during the logarithmic phase increased both the BuGLP mRNA levels and total SOD activity of BuGLP, but decreased the SOD activity bound to the cell wall due to release of most of the SOD activity into the medium. On the other hand, the addition of NaCl to the cells during the stationary phase hardly affected BuGLP mRNA levels or SOD activity levels bound to the cell wall. These results suggest that the induction of BuGLP gene by salt stress is caused by dissociation of BuGLP protein from the cell wall into the medium in the cells during the logarithmic phase.

Keywords: *Barbula unguiculata* — Cell wall reinforcement — Germin-like protein — Mn-SOD — Salt stress.

Abbreviations: CBB, Coomassie brilliant blue; GLP, germin-like protein; SOD, superoxide dismutase; MV, methyl viologen; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR.

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Introduction

Germins are a protein that was first isolated in association with wheat germination. This protein is a water-soluble glycoprotein with oxalate oxidase activity. It forms an oligomer that is highly resistant to proteases and to dissociation by various agents such as heat, SDS and extreme pH (Wei et al. 1998, Carter and Thornburg 2000, Membrel et al. 2000). Proteins with coding elements related to wheat germin such as germin box 1 and box 2 have been found in various land plants and are called germin-like proteins (GLPs) (Dunwell 1998). Some of these GLPs have no oxalate oxidase activity, and the biological function of some GLPs is not comparable with that of germins. Germins and GLPs are suggested to be stress-responsive gene products. For example, accumulation of germin mRNA is up-regulated during the growth of germinating barley seedlings in the presence of NaCl (Hurkman and Tanaka 1996). In contrast, Berna and Bernier (1999) reported that no change in the oxalate oxidase activity of germin was observed in the presence of NaCl in wheat seedlings.

An important response of plants to salt stress is the accelerated production of active oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radical and this production results in oxidative stress (Bellaire et al. 2000, Hernández et al. 2001, Pastori and Foyer 2002). Superoxide dismutase (SOD) (EC 1.15.1.1) is of major importance in protecting living cells from superoxide anion toxicity produced under oxidatively stressed circumstances. We are interested in the SODs of bryophytes, which are considered to have been the first land plant group, and to occupy a critical position in the evolution of other land plants. During the study, an extracellular protein with Mn-SOD activity was isolated from cells of a moss, *Barbula unguiculata*. The protein was identified to be a GLP and designated BuGLP (Yamahara et al. 1999). Although there are some reports that describe extracellular SODs in plants, all of them are CuZn-SODs (Streller and Wingles 1994, Ogawa et al. 1996, Ogawa et al. 1997), and the physiological functions of the SODs have not yet been clarified.

Germins and GLPs are all found to be associated with the cell wall (Lane et al. 1992, Heintzen et al. 1994, Lane 1994, Berna and Bernier 1997), and both SOD and oxalate oxidase produce hydrogen peroxide. These facts have led to some hypotheses about the biological significance of extracellular hydrogen peroxide production; it could serve as a signal for the induction of defense mechanisms (Kovtun et al. 2000); or it could be used in peroxidase-catalyzed cross-linking reactions, lignin production for example, to reinforce the cell wall (Lane et al. 1993, Olson and Varner 1993, Mehdy 1994, Ogawa et al. 1997, Wei et al. 1998). In order to estimate the physiological functions of BuGLP, we examined the effects of salt stress on the expression of BuGLP taking note of its SOD activity. The results indicate that the expression of the *BuGLP* gene was peaked during the exponential phase of growth and first show...
that salt stress caused the dissociation of BuGLP from the cell wall which was accompanied by BuGLP mRNA induction.

**Results**

**Transit peptide of BuGLP**

Subcellular localization of germins and GLPs reveals that they are found associated with the apoplast, which consists of the cell wall and the extracellular fluid (Lane et al. 1992, Heintzen et al. 1994, Lane 1994, Berna and Bernier 1997). Since BuGLP was also first isolated as an extracellular Mn-SOD after washing cells with 0.5 M NaCl (Yamahara et al. 1999), BuGLP probably existed in the apoplast. However, its transit peptide has not yet been identified. Therefore, a full-length cDNA encoding BuGLP was obtained, which was composed of 927 bp, containing the sequence for the BuGLP mature protein. The BuGLP cDNA sequence analyzed by TargetP (Nielsen et al. 1997, Emanuelsson et al. 2000) indicated that there was a transit peptide with 24 amino acids, MYSRSMWTTAVALLVGLVPMAMA, upstream from the amino terminal end of the BuGLP mature protein. The transit peptide contained a short basic amino terminal region, a central hydrophobic region, and a carboxyl terminal region with a small and neutral amino acid, alanine, at positions –3 and –1 relative to the cleavage site. The transit peptide possessed typical features that lead BuGLP to the endoplasmic reticulum and then to extracellular compartments (Emanuelsson et al. 2000).

**BuGLP mRNA expression and accumulation of BuGLP and its SOD activity during cell growth**

The expression of GLP is developmentally regulated; the expression of GLP mRNA in pine was induced in quiescent embryos but not in germinating seeds (Neutelings et al. 1998) and GLP expression oscillated with a circadian rhythm (Ono et al. 1996). Therefore, to analyze the physiological functions of BuGLP, the expression of BuGLP mRNA and the SOD activity of BuGLP protein during growth of *B. unguiculata* cells were studied. Fig. 1 shows the growth of cells, BuGLP mRNA expression, BuGLP protein bound to the cell wall, and SOD activity bound to the cell wall. BuGLP mRNA was abundant during the logarithmic phase of growth (from 3 to 7 d) and decreased during the stationary phase. This demonstrates that BuGLP gene expression depended on the phase of growth. Nevertheless, the SOD activity of BuGLP was relatively high during the stationary phase of growth (Fig. 1C, D). This is in agreement with the former findings (Wei et al. 1998) that *Hordeum vulgare* oxalate oxidase-like protein mRNA level peaked at 18–24 h and subsequently decreased, whereas the protein level was constant from 24 h after inoculation.

The amount of BuGLP demonstrated by Coomassie brilliant blue (CBB) staining (Fig. 1C) was proportional to SOD activity demonstrated by the activity staining and SOD activity assay (Fig. 1D).

**Stability of BuGLP against SDS and heat treatment**

To explain the cause of the inconsistency between BuGLP mRNA levels and the amount of BuGLP and its SOD activities during stationary phase in Fig. 1, the stability of BuGLP...
Moss germin-like protein with Mn-SOD activity was investigated (Fig. 2). The upper and lower arrowheads in Fig. 2 indicate the oligomeric and monomeric forms of BuGLP, respectively. At first BuGLP was confirmed to maintain the oligomeric form and SOD activity on the non-denaturing SDS-PAGE (Fig. 2A, B, lane 1). BuGLP was stable even after treatment at 60°C for 30 min (Fig. 2A, B, lane 4); however, when purified BuGLP was boiled for 5 min (Fig. 2A, B, lane 2), or the crude extracts were held at 80°C for 30 min (Fig. 2A, B, lane 5), they lost most of their activity and their oligomeric form, producing a monomer. These results indicated that BuGLP was stable in 0.1% SDS, and also at 60°C. Although there was only one major band of oligomeric BuGLP with SOD activity (Fig. 2A, B, lane 1), when the BuGLP was boiled two major bands were found; one monomeric BuGLP and the other with a molecular mass of about 37 kDa (Fig. 2A, lane 2). The nature of the latter has not been characterized yet. The relatively high SOD activity of the BuGLP during the stationary phase (Fig. 1) is suggested to be due to the BuGLP accumulation during growth because of its stability.

**Effect of methyl viologen on the expression of BuGLP**

It was unclear whether the physiological functions of BuGLP were related to the activity of dismutating superoxide that was probably generated in the extracellular compartment. Since in cotton callus tissue, methyl viologen (MV) produced a significant increase in the extracellular superoxide levels (Bel-laire et al. 2000), we determined BuGLP mRNA levels and extracellular SOD activities when cells were cultured for 5 d in the presence of MV as the superoxide generator (Fig. 3). Although MV suppressed cell propagation with an increase in MV concentrations (Fig. 3A), BuGLP mRNA levels decreased (Fig. 3B), but the SOD activity of BuGLP protein bound to the cell wall was not affected (Fig. 3C). Furthermore, little SOD activity of BuGLP was released into the medium in the presence of MV (data not shown). These results indicate that MV did not increase in BuGLP mRNA and SOD activity of BuGLP.

**Effects of salt stress on the expression of BuGLP mRNA and accumulation of the SOD activity of BuGLP**

Salt stress causes some changes in plant metabolism to adapt to the environment. Salt stress is composed of osmotic and ionic stresses (Gueta-Dahan et al. 1997, Borsani et al.

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**Fig. 2** Resistance of BuGLP to SDS and heat. (A) CBB staining. (B) SOD activity staining. Lane 1 shows purified BuGLP (5 μg per lane) as an untreated control. Upper and lower arrowheads indicate oligomeric and monomeric forms of BuGLP, respectively. Purified BuGLP (5 μg per lane) was boiled for 5 min in the absence of 2-mercaptoethanol and subjected to SDS-PAGE (lane 2). Lane 3 shows the extracts as an untreated control. Crude extracellular proteins (15 μg per lane, lanes 4 and 5) were heat-treated at 60°C (lane 4) or 80°C (lane 5) for 30 min and subjected to SDS-PAGE. Molecular weights of standards are indicated on the left in kDa.

**Fig. 3** Effects of MV on the expression of BuGLP. Cells were cultured in the presence of 0, 30, 100, and 300 μM MV for 5 d and cell numbers, BuGLP mRNA levels, and the cell wall-bound SOD activities were determined. (A) Cell number per ml of medium. (B) BuGLP mRNA levels demonstrated by Northern blotting. The membrane was exposed to X-ray film for 30 min. Ethidium bromide-stained agarose gel shows loading control. (C) Activities of SOD bound to the cell wall are shown as units per 10^8 cells.
Thus, the effects of NaCl on BuGLP expression were examined in cells cultured in the presence of 100 and 200 mM NaCl. Cells during the logarithmic phase of growth were grown in the respective media for 5 d (Fig. 4). Cell propagation was suppressed by the 100 and 200 mM salt concentrations by about 65% and 85%, respectively (Fig. 4A). Microscopic observation of cells, however, revealed no difference between cells cultured in the presence and absence of salt. BuGLP mRNA levels in cells cultured in the presence of NaCl markedly increased with an increase in the NaCl concentration in the medium (Fig. 4B). The SOD activity bound to the cell wall, however, decreased in cells cultured in the presence of 100 and 200 mM NaCl (Fig. 4C, closed bars). The activity increased in the cultures in the presence of NaCl (Fig. 4C, open bars), and total SOD activity of BuGLP both bound to the cell wall and released into the medium increased under the salt-stressed conditions. The extracellular protein with the SOD activity released into the medium was confirmed to be BuGLP by activity staining after nondenaturing SDS-PAGE (data not shown). These results demonstrate that the salt stress evidently increased BuGLP expression at the transcriptional level. This is consistent with a report that *germin* gene expression is induced by salt stress in barley (Hurkman and Tanaka 1996).

**Effects of salt stress on the expression of BuGLP in cells during the logarithmic phase and the stationary phase**

We were interested in clarifying whether the induction of BuGLP expression is caused directly by salt stress or indirectly by dissociation of BuGLP from the cell wall into the medium. We examined the effects of salt stress on the expression of BuGLP in cells during the logarithmic phase when BuGLP transcript levels were high, and in cells during the stationary phase when they were low as demonstrated in Fig. 1. Cells were cultured for 5 d, and then NaCl was added to a final concentration of 200 mM. BuGLP mRNA levels and SOD activities of BuGLP bound to the cell wall and released into the medium were examined 1 and 2 d after the addition of NaCl (Fig. 5A). Compared to the control cells, the salt-stressed cell propagation was suppressed by about 46% and 68% at 1 and 2 d after the addition of NaCl, respectively (data not shown). Most of the SOD activity was released into the medium by the addition of NaCl and small amounts remained on the cell wall.
BuGLP mRNA levels was increased by the salt stress. These results were almost the same as shown in Fig. 4 where NaCl was present from the start of the culture. On the other hand, when 200 mM NaCl was added to cells which had been cultured for 10 d (Fig. 5B) most of the SOD activity of BuGLP remained on the cell wall, although small amounts were released into the medium by the NaCl treatment. BuGLP mRNA levels were changed little by the salt stress. The addition of NaCl hardly affected the number of cells (data not shown). These results do not indicate that salt stress directly affected the induction of BuGLP expression, but suggest that the increase in the BuGLP mRNA level was caused by dissociation of BuGLP from the cell wall by NaCl treatment in the cells during the logarithmic phase.

Discussion

BuGLP transcripts were abundant in cells during the logarithmic phase of growth (Fig. 1B). The addition of NaCl to the cells during the logarithmic phase induced the release of BuGLP protein from the cell wall, and the BuGLP mRNA expression increased in such a way that it apparently compensated for the loss by the release (Fig. 4, 5A). When BuGLP was kept bound to the cell wall during the stationary phase of growth or by the addition of MV, BuGLP mRNA levels were low (Fig. 3, 5B). These results suggest that BuGLP plays a role in the process of cell propagation by contributing to extracellular matrix synthesis by hydrogen peroxide produced by BuGLP.

The addition of MV caused no decrease in the SOD activity bound to the cell wall, although it caused a decrease in BuGLP mRNA levels (Fig. 3). This may be consistent with the report on barley GLP, which has no oxalate oxidase activity, where hydrogen peroxide treatment as well as pathogen infection caused a decrease in the mRNA abundance, although these treatments caused the GLP to become insoluble and resulted in a tight association with the cell wall (Valletz-Bindschedler et al. 1998). Furthermore, in plant disease resistance hydrogen peroxide generated from superoxide by an extracellular CuZn-SOD caused the cell wall proteins to become insoluble by cross-linking, to render the cell walls more refractory to pathogen ingress (Brisson et al. 1994, Schweizer et al. 1995). Our results suggest that the MV treatment might cause a tight association of BuGLP protein with the cell wall by cross-linking.

Little is known about how the signal of the dissociation of BuGLP by salt stress is perceived and transduced to induction of BuGLP mRNA. Since hydrogen peroxide generated by the action of apoplastic SODs is shown to play a role as a central signaling molecule in several signal transduction pathways (Kovtun et al. 2000, Pastori and Foyer 2002), it is possible that hydrogen peroxide generated by BuGLP may be related to the signal transduction. The dissociation of BuGLP from the cell wall by salt stress may lead to the reduction of hydrogen peroxide for signaling and it finally causes induction of BuGLP expression via hydrogen peroxide related signal transduction. This is in agreement with the result that MV treatment was accompanied by the reduction of BuGLP mRNA (Fig. 3), that is, hydrogen peroxide is more likely to be generated from superoxide produced by BuGLP in the MV treatment.

Materials and Methods

Plant material and culture conditions

Cells of B. unguiculata were propagated by shaking on a gyratory shaker at 110 rpm at 25°C in the light as described previously (Yamahara et al. 1999). In the salt stress experiments, cells were grown in the presence of NaCl at an initial concentration of 100 or 200 mM. To investigate the effects of salt stress on cells during the logarithmic phase and the stationary phase, fresh NA-MS medium containing 4.6 M NaCl was added up to 200 mM to the cell broth which had been cultured for 5 and 10 d, respectively. The effects of MV were investigated by growing the cells for 5 d in the presence of MV at an initial concentration of 30, 100, or 300 μM.

RNA extraction and cloning of full-length cDNA of the BuGLP gene

Total RNA was isolated from cells based on the standard guanidine isothiocyanate extraction and cesium chloride ultracentrifugation method (Chirgwin et al. 1979) and treated with deoxynucleobonuclease (RT Grade, Nippon gene). Partial cDNA of BuGLP was cloned previously (Yamahara et al. 1999). For 5’RACE-PCR, the first strand cDNAs were synthesized using a gene specific primer 5’-TCGTTAGGGCCG-GGGAACCTT-3’ and tailed with poly dA by terminal deoxynucleotidyl transferase (Wako). The second cDNAs were synthesized using an oligo (dT) containing adapter primer (3’RACE system kit, GIBCO BRL). The 5’RACE-PCR was performed using the gene specific primer (described above) and the primer Abbrided Universal Amplification Primer (3’RACE system kit, GIBCO BRL). For cloning full-length cDNA, RT-PCR was performed using 3’RACE system kit (GIBCO BRL), and primers 5’-GATTCCATCATCAGGCTAAG-3’ and 5’-GCCCCATGAAAGGGAAAGAATT-3’. PCR products extracted from agarose gel were cloned into pGEM-T EASY vector (Promega) and sequenced using a Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and with a DNA sequencer (model 373A, PE Applied Biosystems).

Northern blot analysis

The BuGLP DNA probe used for hybridization was labeled with digoxigenin (DIG) using a DIG DNA Labeling and Detection Kit (Roche diagnostics) by PCR. Template DNA was a Psrl fragment excised from a pGEM-T EASY vector containing BuGLP full-length cDNA as the insert DNA, and the primers were 5’-TTCTGCAGTGCGAGACACCA-3’ and 5’-GCCCATGAAAGGGAAAGAATT-3’. The DIG labeled BuGLP DNA probe contains an open reading frame and 3’ untranslated region of BuGLP cDNA.

Denatured total RNA (20 μg) was electrophoresed on 1% (w/v) agarose gels containing 15% (v/v) formaldehyde and transferred onto Hybond-N+ membranes (Amersham Pharamacia) using 20× SSC. Hybridization was performed at 45°C with a DIG-labeled BuGLP DNA probe and washed twice in 2× SSC, 0.1% SDS for 5 min at room temperature and twice 0.1× SSC, 0.1% SDS for 15 min at 65°C. Hybridization signals were detected with Anti-DIG-AP conjugate and CSP-Star (Roche diagnostics). Hybridization and detection were performed according to manufacturer’s instructions. All experiments were repeated at least twice with essentially similar results and a typical one is shown.
Preparation of extracellular protein

To extract extracellular proteins bound to the cell wall, cells were suspended in 500 mM NaCl, followed by gentle stirring for 30 min at 4°C, and filtered with suction. Ammonium sulfate was added to the extracellular filtrate to bring it up to 90% of saturation. To obtain the proteins released into the medium, cells were first removed from the medium, and ammonium sulfate was added to the medium to bring it up to 90% of saturation. After centrifugation the precipitate was dissolved in a minimum volume of 20 mM Tris-HCl (pH 8.0), and dialyzed against the same buffer.

Assay for SOD activity

SOD assays were carried out as described previously (Yamahara et al. 1999).

For SOD activity staining, SDS-PAGE was performed according to the methods of Laemmli (1970) except the sample buffer did not contain 2-mercaptoethanol, and samples were not treated with heat to denature them. Following electrophoresis, the 15% SDS-containing gel was washed as described by Carter and Thornburg (2000). Then, the gel was stained by the riboflavin/nitro blue tetrazolium method described by Beauchamp and Fridovich (1971). All experiments were repeated at least twice with essentially similar results and one that is typical is shown.

Measurement of cell number

To investigate cell growth, cells grown in NA-MS medium were collected in a 1.5 ml test tube. After centrifugation at 70g, the supernatant was removed and then an equal volume of isotonic solution, containing 0.6 M sorbitol and 5 mM MES-NaOH (pH 5.8), was added. This step was repeated three times. After the final centrifugation at 70g, the supernatant was removed and then an equal volume of cellu-lase solution, containing 0.6 M sorbitol, 5 mM MES-NaOH pH 5.8, 1% (w/v) Onozuka RS (Yakult), and 0.1% (w/v) Pectolase Y-23 (Kikkoman), was added. Cellulase treatment was performed at 37°C for 30 min with gentle mixing every 5 min. Cellulase-treated cells were counted using a hemocytometer. Final values are averages of at least duplicate samples which were counted at least 15 times per sample.

Preparation of BuGLP

Purified BuGLP was obtained as described previously (Yamahara et al. 1999).

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References


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