広島大学学位請求論文

Germin-like proteins of mosses: their physiological roles and phylogenetically characteristic positions in plants

（コケ植物の germin-like protein：
系統学的特徴と生理機能の解析）

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主　論　文

Germin-like proteins of mosses: their physiological roles and phylogenetically characteristic positions in plants

Masaru Nakata

Laboratory of Plant Molecular Physiology

Department of Biological Science

Graduate School of Science

Hiroshima University

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CONTENTS

ABBREVIATIONS

× 1 SSC, 150 mM NaCl and 15 mM sodium citrate (pH 7.0);

b(p), base (pair);

CBB, Coomassie brilliant blue;

cDNA, complementary DNA;

CTAB, cetyltrimethylammonium bromide;

Da, dalton;

DIG, digoxigenin;

EST, expressed sequence tag;

GLP, germin-like protein;

H_2O_2, hydrogen peroxide;

MES, 2-morpholinoethanesulfonic acid;

mRNA, messenger RNA;

MS-medium, Murashige and Skoog's medium;

MV, methyl viologen;

NJ, neighbor-joining;

O_2^-, superoxide

OXO, oxalate oxidase;

PAGE, polyacrylamide gel electrophoresis;

RACE, rapid amplification of cDNA ends;

ROS, reactive oxygen species;

rpm, revolutions per minute;

rRNA, ribosomal RNA;

RT-PCR, reverse transcription-PCR;

S, Svedberg unit;

SDS, sodium dodecyl sulfate;

SOD, superoxide dismutase;

Tris, tris(hydroxymethyl) aminomethane;

PREFACE

Germin is a protein that was first isolated in association with wheat germination (Thompson and Lane, 1980). This protein is localized to the cell wall and is a water-soluble glycoprotein with oxalate oxidase (OXO) activity that converts oxalate to carbon dioxide and hydrogen peroxide (H_2O_2) (Lane et al., 1993). Proteins homologous to germin widely distribute among land plants and designated germin-like protein (GLP). Numerous literatures reported that the expression of germins and GLPs are induced by biotic and abiotic stresses such as pathogen, wound and salt (Hurkman and Tanaka, 1996; Berna and Bernier, 1999; Park et al., 2004; Tabuchi et al., 2003), and plant hormones like auxin and cytokinin (Hurkman and Tanaka, 1996; Ohmiya et al., 1998; Caliskan et al., 2004), and are also up-regulated during cell division and elongation (Mathieu et al., 2003; Caliskan, 2004; Kim et al., 2004)

There are two problems to clarify physiological roles of germins and GLPs. First, germins have OXO activity, although the enzymatic activity of GLPs had never been clarified. Degradation of oxalate and generation of H_2O_2 by OXO activity of germin are important for defense response against pathogen infection. H_2O_2 generated in apoplast serves as a multifunctional molecule for signal for the induction of defense mechanisms (Kovtun et al. 2000), cell wall modification (Lewis and Yamamoto, 1990; Schopfer et al., 2002; Liszkay et al., 2004; Passardi et al., 2004), and direct antimicrobial action (Lamb and Dixon, 1997). These evidences facilitated the elucidation of physiological roles of germin. Although the expression patterns of some GLPs were similar to those of germins, there was no clue to infer the enzymatic activity

of GLPs, much less their physiological roles. Second, germins and GLPs constitute a large, diverse and redundant gene family in the genome. Functional redundancy of germin and GLP family may bury a mutation in *GLP* gene by complementation of other GLPs, resulting in prevention against uncovering their physiological function using genetics. In fact, mutants of germin or GLP have not been reported. Phylogenetic analysis of germins and GLPs demonstrated that germins and GLPs in higher plants were divided into five clades (Carter and Thornburg. 2000). Germins with OXO activity grouped into a subfamily named true germin subfamily and GLPs divided into other four subfamilies.

Yamahara et al. (1999) isolated a novel GLP with manganese-containing superoxide dismutase (Mn-SOD) activity from a moss, *Barbula unguiculata* and designated BuGLP. BuGLP possesses all characteristic features of GLP such as three conserved domains and a polysaccharide-binding site. The discovery was the first report that demonstrates GLP with enzymatic activity, and triggered the identification of a germin and some GLPs as Mn-SOD. To date, barley germin (Woo et al., 2000) and GLP from tobacco (Carter and Thornburg, 2000), wheat (Segarra et al., 2003), and halophyte *Atriplex lentiformis* (Tabuchi et al., 2003) have been reported to have Mn-SOD activity. These germin and GLPs are spread over several subfamilies in the phylogenetic tree, suggesting that acquisition of GLPs with Mn-SOD activity occurred in quite early stage of diversification of GLP family. Elucidation of physiological roles of GLP taking note of its Mn-SOD activity possibly links to understanding of functions of entire GLP family. On the other hand, barley GLP with ADP-glucose pyrophosphatase /phosphodiesterase (AGPPase) activity was isolated (Rodríguez-López et al., 2001). This finding suggests the possibility of the existence of GLPs with other

enzymatic activity in the family. Therefore, the comprehensive analysis of GLP family as well as the analysis based on Mn-SOD activity is required for complete understanding of this family.

I have been interested in physiological roles of GLP and how GLP family has been acquired and diversified during the evolution of plants. Bryophytes have been believed to be the first land plant group (Kenrick and Crane, 1997; Qiu and Palmer, 1999) and occupy an important position to discuss the origin and diversification of GLP family because the family is observed only in land plants. However, the analysis focused on GLP or GLP family in bryophytes has not been carried out.

The aim of this study is to clarify the physiological roles of GLPs in bryophytes. In order to estimate the physiological roles of BuGLP, *B. unguiculata* cultured cells were used for the expression analysis of *BuGLP*. Cultured cells are useful in respect to altering culture conditions, but it is difficult to obtain more detailed information about localization of BuGLP in plant and to perform genetic analysis. *Physcomitrella patens* subsp. *patens* was used for further analysis. *P. patens* is a widely used model plant because it has relatively small genome size and the highest ratio of homologous recombination to nonhomologous recombination of all land plants (Schaefer, 2002). The ability to generate gene disruptant by homologous recombination is a strong tool to analyze the physiological roles of GLPs. Deposition of numerous number of EST clones of *P. patens* in DNA database (Rensing et al., 2002; Nishiyama et al., 2003) is appropriate to comprehensive analysis of GLP family. BuGLP-like extracellular Mn-SOD activity was detected from cell wall extract of *P. patens*, suggesting existence of GLP with Mn-SOD activity in *P. patens*.

In chapter I, to characterize the expression of *BuGLP*, I examined the effects of

salt stress and H_2O_2 on the expression of BuGLP taking note of its SOD activity. Salt and osmotic stress induces the expression of *BuGLP* and dissociation of BuGLP from cell wall. Salt and osmotic stress signals, and H_2O_2 and ABA signals are transmitted through independent pathway and result in opposite regulation of *BuGLP* expression.

In chapter II, I performed a phylogenetic analysis of the GLP family of *P. patens*, proposing two novel clades designated bryophyte subfamily 1 and 2, which are clearly distinguishable from the clades of higher plant GLPs. One of the most remarkable features of bryophyte subfamily 1 is a lack of 12 amino acid containing two cysteines at conserved positions in all germins and GLPs. Moreover, I revealed that PpGLP6 had extracellular Mn-SOD activity.

CHAPTER I

Salt stress-induced and hydrogen peroxide-repressed expression of a germin-like protein with Mn-SOD activity in a moss, *Barbula unguiculata*

Abstract

Germin and germin-like protein (GLP) widely distribute among land plants, but their physiological roles have not been clarified. We isolated a GLP with manganese-superoxide dismutase (Mn-SOD) activity (BuGLP) from a moss, *Barbula unguiculata*. I examined the expression patterns of BuGLP taking note of its SOD activity under several growth conditions. *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 during growth. The addition of NaCl to the cells during the logarithmic phase increased the *BuGLP* mRNA levels but decreased BuGLP bound to cell wall due to the release of most of BuGLP into the medium. On the other hand, salt stress during the stationary phase hardly affected *BuGLP* mRNA levels and more than half of BuGLP was maintained on cell wall. Although salt stress closely relates to production of reactive oxygen species, the addition of hydrogen peroxide (H_2O_2) repressed the accumulation of *BuGLP* mRNA. H_2O_2 partly repressed NaCl-induced accumulation of *BuGLP* mRNA, suggesting that salt stress and H_2O_2 signals are transmitted through independent pathways and regulate *BuGLP* transcription oppositely.

Introduction

Germin is a protein which was first isolated in association with wheat germination. This protein is a water-soluble glycoprotein with oxalate oxidase (OXO) 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; Membré et al., 2000). Proteins related to wheat germin have been found in various land plants and are called germin-like proteins (GLPs) (Dunwell, 1998). The physiological roles of germin have been partly revealed that germins are likely to play an important role in several aspects of plant growth, stress response, and defense against pathogen infection. Overexpression of germin with OXO activity caused elevation of defense responses and increased resistance against pathogen (Liang et al., 2001; Burke and Rieseberg, 2003; Hu et al., 2003). These effects are thought to arise from hydrogen peroxide (H_2O_2) generating ability of OXO. However, as shown in phylogenetic analysis of germin and GLP family (Carter and Thornburg., 2000; Khuri et al., 2001), germins occupy only a small part of their family and most of other GLP family members have no enzymatic activity, and the biological function of GLPs is not comparable with that of germins. Although numerous researchers have tried to clarify their physiological roles, none of their functions have been revealed yet.

Salt stress composed of ionic and osmotic stresses and these signals are transmitted independently (Zhu, 2002). An important response of plants to salt stress is the accelerated production of reactive oxygen species (ROS) such as superoxide (O_2^-), H_2O_2, and the hydroxyl radical and the production results in oxidative stress (Bellaire et

al., 2000; Hernández et al., 2001; Pastori and Foyer, 2002). ROS has been considered as a toxic cellular metabolite. However, it functions as a signaling molecule that mediates responses to various stimuli, and modulates the expression of a number of genes, including those encoding antioxidant enzymes and modulators of ROS production (Neill et al., 2002; Mittler et al., 2004). Under salt-stressed condition, the expressions of ROS detoxification enzymes such as superoxide dismutase (SOD) are induced (Gueta-Dahan et al., 1997). Plasma membrane-localized NADPH oxidase that is homologous to $gp91^{phox}$, plasma membrane protein in mammalian neutrophils, is responsible for the production of O_2^- in apoplast in higher plants (Torres et al., 2002). In addition to NADPH oxidase, pH-dependent cell wall peroxidases, germins (OXO), and amine oxidases have been proposed to generate ROS in apoplast (Mittler et al., 2004).

Abscisic acid (ABA) controls important cellular processes and plays a protective role in response to abiotic stresses including drought, salinity and cold (Finkelstein et al., 2002; Zhu, 2002). ABA causes stomatal closing under drought conditions. Cytosolic Ca^{2+} mediates the ABA signaling as a second messenger in guard cells (Yang and Poovaiah, 2003). Increases of ABA-induced cytosolic Ca^{2+} concentration are mediated by Ca^{2+} influx through plasma membrane-localized Ca^{2+} channels and Ca^{2+} release from internal stores (MacRobbie, 2000; Pei et al., 2000). These Ca^{2+} channels were demonstrated to be stimulated by H_2O_2, suggesting that H_2O_2 plays an important role in increase of cytosolic Ca^{2+} concentration in ABA signaling in guard cells (Pei et al., 2000; Himmelbach et al., 2003; Kwak et al., 2003).

Bryophytes are considered to have been the first land plant group, and to occupy a

critical position in the evolution of land plants (Kenrick and Crane, 1997). Because it is speculated that bryophytes were exposed to highly oxidatively stressed circumstances and developed mechanisms against oxidative stress during adaptation to terrestrial life, I am interested in the SODs of bryophytes. SOD is of major importance in protecting living cells from O_2^- toxicity produced under oxidative environment. During the study, an extracellular protein with Mn-SOD activity was isolated from cells of a moss, *Barbula unguiculata*. The protein was identified as a GLP and designated BuGLP (Yamahara et al., 1999). GLPs with Mn-SOD activity were identified from several higher plants after that (Carter and Thornburg, 2000; Woo et al., 2000; Segarra et al., 2003; Tabuchi et al., 2003). Because H_2O_2 productive ability is common to both SOD and OXO, these discoveries lead to a speculation that the biological significance of GLP is the same as that of germin by extracellular H_2O_2 production. It could serve as a signal for the induction of defense mechanisms (Kovtun et al., 2000) or could be used in the modification of cell wall (Olson and Varner, 1993; Mehdy, 1994; Ogawa et al., 1997; Wei et al., 1998; Mittler et al., 2004).

In this chapter, I performed characterization of *BuGLP* expression. The expression of *BuGLP* was induced under salt-stressed condition accompanied by detachment of BuGLP from cell wall, whereas H_2O_2, ABA, influx of Ca^{2+} into cytosol decreased its mRNA. This suggests that salt and H_2O_2 signals are transmitted through independent signaling pathway in *B. unguiculata*.

Materials and Methods

Plant material and culture conditions

Cells of *Barbula 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). 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 days, respectively. The effects of NaCl, MV, and mannitol were investigated by growing the cells for 5 days in the presence of the compounds at concentrations shown in figures and figure legends. H_2O_2, diphenyleneiodonium (DPI), ABA, and A23187 were added to 5 days cultured cells and the cells were collected 1 day after the addition.

RNA extraction and Northern hybridization analysis

Total RNA was isolated from cells based on the standard guanidine isothiocyanate extraction and cesium chloride ultracentrifugation method (Chirgwin et al., 1979). 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 Pharmacia) using 20 × SSC. Hybridization was performed at 45°C with a digoxigenin (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 CDP-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.

DIG-labeled DNA probe used for the hybridization was labeled using a DIG DNA Labeling and Detection Kit (Roche diagnostics) by PCR. Template DNA was a *Pst*I fragment excised from a pGEM-T EASY vector containing *BuGLP* full-length cDNA as the insert DNA (see below), and the primers were 5'-TTCTGCGTGCGAGACACCA-3' and 5'-GCCCATGAAAGGGAAGAATT-3'. The DIG labeled *BuGLP* DNA probe contains an open reading frame and 3' untranslated region of *BuGLP* cDNA.

Cloning of full-length cDNA of BuGLP *gene*

Five μg of total RNA was treated with deoxyribonuclease (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'-TTCTTGAGGCCGGCGAACTT-3' and tailed with poly dA by terminal deoxynucleotidyl transferase (Wako). The second strand 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 Abridged Universal Amplification Primer (3'RACE system kit, GIBCO BRL).

For cloning of full-length *BuGLP* cDNA, RT-PCR was performed using primers 5'-GATTCATCATCAGCCTCAAG-3' and 5'-GCCCATGAAAGGGAAGAATT-3', and first strand cDNAs synthesized with oligo(dT) primer. 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).

Preparation of extracellular protein

To extract extracellular proteins bound to cell wall, cells cultured in liquid medium 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 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 typical is shown. Quantification of the SOD activity in the gel was performed using NIH image 1.63 software.

Measurement of cell number

To investigate cell growth, cells grown in liquid medium were collected in a 1.5 ml test tube. After centrifugation at 70 × *g*, 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 70 × *g*, the supernatant was removed and then an equal volume of cellulase solution, containing 0.6 M sorbitol, 5 mM MES-NaOH pH 5.8, 1% (w/v) Onozuka RS (Yakult), and 0.1% (w/v) Pectoriase 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 purified BuGLP

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

Results

Transit peptide of BuGLP

Subcellular localization of germins and GLPs reveals that they are found associated with the apoplast, which consists of 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 localizes in apoplast. However, its transit peptide has not yet been identified. Therefore, a full-length cDNA encoding *BuGLP* was cloned, which was composed of 927 bp. 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, MYSRSMWTTVAVLLVVGLVPMAMA, upstream from the amino terminal end of the BuGLP mature protein. The putative transit peptide of BuGLP possessed typical features that consists of 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. This suggests apoplastic localization of BuGLP.

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

The expression of GLP is developmentally regulated in higher plants; the expression of *GLP* mRNA in pine was detected in quiescent embryos but not in germinating seeds (Neutelings et al., 1998) and GLP expression oscillated with a circadian rhythm (Ono et al., 1996). To examine whether the expression of *BuGLP* undergoes such developmental regulation in cultured cells of *B. unguiculata*, the expression of *BuGLP* mRNA and the SOD activity of BuGLP protein during growth were studied. Figure 1 shows the growth pattern of cells, *BuGLP* mRNA expression, BuGLP protein bound to cell wall, and SOD activity bound to cell wall. *BuGLP* mRNA was abundant during the logarithmic phase of growth (from 3 to 5 days) and

decreased during the stationary phase. This demonstrates that *BuGLP* gene expression depended on the phase of growth. Nevertheless, the SOD activity of BuGLP hardly varied during 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 to 24 hours and subsequently decreased, whereas the protein level was constant from 24 hours after inoculation.

The amount of BuGLP demonstrated by Coomassie brilliant blue (CBB) staining (Fig. 1C) was proportional to SOD activity shown by the activity staining and SOD activity assay (Fig. 1D). Because no other SOD activity except BuGLP was detected in SOD activity staining shown in Fig. 2, the SOD activity in the extracellular crude extract was largely derived from BuGLP.

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 was investigated (Fig. 2). Purified BuGLP or extracellular crude extract was subjected to SDS-PAGE without heat denature. Heat stability of BuGLP was evaluated by retention of oligomeric form and SOD activity. The upper and lower arrowheads in Fig. 2 indicate the oligomeric and monomeric forms of BuGLP, respectively. BuGLP was confirmed to maintain the oligomeric form and SOD activity on the nondenaturing SDS-PAGE (Fig. 2A, B, lanes 1, 3). 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 maintained SOD activity and oligomeric form even in the presence of 0.1% SDS, and also at 60°C, whereas BuGLP denatured and lost SOD activity at 80°C. Therefore, the unexpected high SOD activity of the BuGLP during the stationary phase, when the level of *BuGLP* mRNA was decreased (Fig. 1), is suggested to be due to the stability of BuGLP.

Although there was only one major band of oligomeric form of BuGLP in lane of nondenatured purified BuGLP (Fig. 2A, B, lane 1), when the purified BuGLP was boiled two major bands were found in the electrophoresis; one monomeric BuGLP and the other with a molecular mass of about 37 kDa (Fig. 2A, lane 2). The partial amino acid sequencing by peptide mass fingerprint revealed that the 37 kDa protein was similar to *Pseudomonas aeruginosa* alkaline phosphatase, which is homologous to human protein called DING protein. This is consistent with the observation that some GLPs interact with plant homolog of DING protein (Berna et al., 2002).

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

Salt stress in plants causes the changes in the metabolism to adapt their environment. The effects of NaCl on *BuGLP* expression were examined in cells cultured in the presence of 100 and 200 mM NaCl. Cells were treated with NaCl by grown in the NaCl containing medium for 5 days (Fig. 3). Cell propagation was suppressed by the 100 and 200 mM salt concentrations by about 65% and 85%, respectively (Fig. 3A). Under microscopic observation, cellulase treated cells for

counting cell number were withered probably because of plasmolysis in the presence of NaCl. *BuGLP* mRNA levels in cells cultured in the presence of NaCl markedly increased with an increase in the NaCl concentration in the medium (Fig. 3B). The SOD activity bound to cell wall, however, decreased in cells cultured in the presence of 100 and 200 mM NaCl (Fig. 3C, closed bars). This is inconsistent with the amount of *BuGLP* mRNA. Because BuGLP is extracted from cell wall by treatment with 0.5 M NaCl (see materials and methods), I examined the amount of SOD activity of BuGLP eluted into the culture medium. A large amount of BuGLP and SOD activity was detected from medium-eluted protein fraction in the presence of NaCl (Fig. 3C, open bars), and total SOD activity of BuGLP both of cell wall and the medium increased under the salt-stressed conditions. The extracellular protein with the SOD activity released into the medium was confirmed to be derived from BuGLP by activity staining after nondenaturing SDS-PAGE (data not shown). These results demonstrate that the salt stress evidently increased *BuGLP* expression at mRNA level. This is consistent with a report that *germin* gene expression is induced by salt stress in barley (Hurkman and Tanaka, 1996).

Osmotic stress induced expression of BuGLP

Salt stress consists of ionic and osmotic stresses (Zhu, 2002). To determine whether ionic or osmotic stress is the cause of salt-induced expression of *BuGLP*, I examined the effect of mannitol. Cells were cultured for 5 days in the medium containing 400 mM mannitol and the amount of *BuGLP* mRNA and SOD activity were analyzed (Fig. 4). Apparent effect of the addition of mannitol for cell growth was not

observed (data not shown). The addition of mannitol increased the amount of *BuGLP* mRNA (Fig. 4A). High concentration of mannitol, which induces non-ionic osmotic stress, caused release of BuGLP from cell wall into medium, although the effects were not so obvious as salt stress (Fig. 4B). These results suggested that non-ionic osmotic stress is sufficient for the induction of *BuGLP* and ionic stress is required for the maximum release of BuGLP from cell wall.

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

I am interested in whether the induction of *BuGLP* expression and dissociation of BuGLP from cell wall are associated. I 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 days, and then NaCl was added up to 200 mM. *BuGLP* mRNA levels and SOD activities of BuGLP were examined 1 and 2 days after the addition of NaCl (Fig. 5A and C). Compared to the control cells, the salt-stressed cell propagation was suppressed by about 46% and 68% at 1 and 2 days 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 cell wall. *BuGLP* mRNA levels was increased by the salt stress. These results were almost the same as shown in Fig. 3 where NaCl was present from the start of the culture. On the other hand, when 200 mM NaCl was added to stationary phase cells, which had been cultured for 10 days (Fig. 5B and D), although BuGLP released into the medium

by NaCl treatment was not negligible, large part of BuGLP protein and SOD activity remained on cell wall. Salt stress did not affect the *BuGLP* mRNA level in cells of stationary phase. The addition of NaCl hardly affected the number of cells (data not shown). These results indicate that there are no relations between increase of *BuGLP* mRNA under salt stress and dissociation of BuGLP from cell wall.

Although *BuGLP* mRNA level was not induced in cells during stationary phase under salt-stressed condition, the total amount of SOD activity of BuGLP seems to be increased (Fig. 5D). The reason of this increase is unknown.

Effect of H_2O_2 on the accumulation of BuGLP *mRNA*

Salt stress accelerates production of ROS in higher plants (Pastori and Foyer, 2002; Zhu, 2002). H_2O_2 plays important roles as a signaling molecule and regulates expression of various genes. The effect of H_2O_2 was determined in the cells of logarithmic phase of growth. The addition of H_2O_2 to 5 days cultured cells decreased the amount of *BuGLP* mRNA (Fig. 6A). On the other hand, the addition of KI, quencher of H_2O_2, increased it (Fig. 6B). Both effects depend on their concentrations. These results indicate that the concentration of H_2O_2 in apoplasts negatively affects the amount of *BuGLP* mRNA.

Although salt stress induces ROS production in higher plants, NaCl and H_2O_2 caused opposite effects on the accumulation of *BuGLP* mRNA in *B. unguiculata.* I was interested in the relationship between the effects of NaCl and H_2O_2 on the expression of *BuGLP*. Because a barley GLP insolubilized by H_2O_2 treatment as a result of oxidative cross-linking to the cell wall (Vallelian-Bindschedler et al., 1998),

H_2O_2 treatment may cause cross-linking of BuGLP to the cell wall and results in repression of detachment BuGLP under salt-stressed condition. To examine the relation of salt stress and H_2O_2 treatment on the expression of *BuGLP*, both NaCl and H_2O_2 were added to the cells of logarithmic phase of growth. Although the addition of H_2O_2 hardly affected to cell growth, NaCl and both NaCl and H_2O_2 suppressed it (Fig. 7A). The addition of NaCl or H_2O_2 caused increase or decrease of the amount of *BuGLP* mRNA, respectively (Fig. 7B). SOD activity and the amount of BuGLP bound to cell wall and amount eluted into medium were not affected by the addition of H_2O_2 (Fig. 7C). When both NaCl and H_2O_2 were added, *BuGLP* mRNA was increased compared to that of control but was decreased compared to that of NaCl-treated cells, whereas NaCl-induced detachment of BuGLP from cell wall was hardly suppressed by the addition of H_2O_2, indicating that BuGLP is not cross-linked to cell wall by H_2O_2. These results suggest that H_2O_2 and NaCl affect on the expression of *BuGLP* through independent pathway.

Effect of methyl viologen on the expression of BuGLP

Exogenous H_2O_2 caused repression of the expression of *BuGLP*. To examine the effect of ROS generated by the cells, the effect of methyl viologen (MV) on the expression of *BuGLP* was determined in the cells cultured for 5 days in the MV containing medium. Because MV produced a significant increase in the extracellular O_2^- levels in cotton callus cells (Bellaire et al., 2000), MV would be expected to induce O_2^- production in *B. unguiculata* cultured cell. MV suppressed cell propagation mildly with an increase in the concentrations of MV and decreased *BuGLP* mRNA level, but

the SOD activity of BuGLP protein bound to cell wall was not affected (Fig. 8). BuGLP were hardly released into the medium in the presence of MV (data not shown). These results indicate that MV repressed the expression of *BuGLP* and did not cause detachment of BuGLP from cell wall. This is consistent with the results of the addition of H_2O_2 (Figs, 6 and 7), suggesting that MV provokes O_2^- production in *B. unguiculata* cultured cell. The effects of MV and H_2O_2 demonstrate that ROS in apoplastic space suppress *BuGLP* transcription.

Effect of NADPH oxidase inhibitor

ROS generation in apoplast is largely dependent on enzymatic reactions. Most characterized ROS producing enzyme in higher plants is a plasma membrane-localized NADPH oxidase, which transfers an electron from cytosolic NADPH to apoplastic molecular oxygen for generation of O_2^- (Sagi and Fluhr, 2001; Yoshioka et al., 2003). To determine the effect of inhibition of NADPH oxidase on the expression of *BuGLP*, diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, was used. If NADPH oxidase is involved in O_2^- production in *B. unguiculata*, the addition of DPI should increase *BuGLP* mRNA as the addition of KI, a quencher of H_2O_2 (Fig. 6B). The addition of DPI caused reduction of *BuGLP* mRNA dose-dependent manner (Fig. 9A). Dimethyl sulfoxide, solvent of DPI, treated cells were used as control (Fig. 9A, 0 μM). This result suggests that the main enzyme contributing to apoplastic O_2^- production in *B. unguiculata* is not NADPH oxidase. Alternatively, DPI could not inhibit the function of NADPH oxidase in the moss. However, the reason why *BuGLP* mRNA was reduced by the addition of DPI is not evident at present.

Effect of ABA on the accumulation of BuGLP *mRNA*

Salt and osmotic stresses closely relate to ABA synthesis in higher plants. ABA activates the synthesis of H_2O_2 in guard cells and the subsequent signaling is mediated by H_2O_2 (Pei et al., 2000). Genome-wide gene expression study has revealed ABA decreased the transcripts of an *Arabidopsis* GLP (Hoth et al., 2002). I investigated the effect of ABA on the accumulation of *BuGLP* mRNA. The addition of ABA decreased the amount of *BuGLP* mRNA (Fig. 9B). This result is in accordance with the effect of H_2O_2, suggesting that the involvement of H_2O_2 in ABA signal transduction pathway in moss as well as in guard cells of higher plants.

Ca^{2+} influx reduces the amount of BuGLP *mRNA*

Ca^{2+} serves as an intracellular messenger in many phytohormone signaling processes, including ABA responses in which the Ca^{2+} influx is triggered by secondary messengers such as H_2O_2 (Neill et al., 2002; Himmelbach et al., 2003). Elicitor, ABA, and H_2O_2 signaling converge on influx of Ca^{2+} from apoplast through plasma membrane-localized Ca^{2+} channels in guard cells of higher plants (Klüsener et al., 2002). Ca^{2+} ionophores have been used for studying involvement of Ca^{2+} in ABA signaling in plants (Sheen, 1996; Takezawa, 1999). To examine whether influx of Ca^{2+} into cytosol affects on the expression of *BuGLP*, I performed pharmacological analysis. Selective Ca^{2+} ionophore A23187 causes influx of Ca^{2+} into cytosol from apoplast and vacuole, an intercellular storage of Ca^{2+}, and is effective to increase cytosolic Ca^{2+} concentration. Figure 10 shows the effect of A23187 on the expression of *BuGLP*.

The addition of A23187 reduced the amount of *BuGLP* mRNA, indicating that increase of cytosolic Ca^{2+} concentration represses accumulation of *BuGLP* transcripts. These results demonstrate that Ca^{2+} dependent pathway is involved in regulation of the expression of BuGLP.

Discussion

The effects of ABA, H_2O_2, and Ca^{2+} ionophore on the expression of *BuGLP* were investigated (Figs. 6, 9 and 10). These treatments reduced the amount of *BuGLP* mRNA. ABA signal is transmitted through generation of H_2O_2 in apoplast and increase of cytosolic Ca^{2+} concentration in higher plants (Himmelbach et al., 2003). Because various ABA responses and the role of Ca^{2+} as a second messenger are also conserved in a moss *Physcomitrella patens* (Knight et al., 1995; Minami et al., 2003; Takezawa, 2003), it would be reasonable to postulate that H_2O_2 and Ca^{2+} influx are also involved in ABA signaling pathway in *B. unguiculata.*

Although osmotic stress-induced ABA accumulation is well known in higher plants, ABA and osmotic stress caused opposite effects on the expression of *BuGLP* mRNA in *B. unguiculata* cultured cells. This suggests that ABA signal and osmotic stress signal in regulation of *BuGLP* are transmitted through different pathway as shown in Fig. 11. Osmotic stress signal could be transmitted through ABA-independent pathway and up-regulate the expression of *BuGLP*. ABA-dependent and ABA-independent gene expression in response to abiotic stress is reported in higher plants (Shinozaki et al., 2003). Regulation of gene expression at mRNA levels is

divided into two steps, transcriptional and post-transcriptional regulations. ABA-responsive element (ABRE) and drought-responsive element (DRE) are *cis*-acting elements that function in ABA-dependent and ABA-independent transcriptional regulation, respectively. A number of homologues of salt stress-related proteins of higher plants, including transcription factors and protein kinases, are induced by the addition of NaCl in *P. patens* (Frank et al., in press). Because both ABRE and DRE of higher plants are thought to be functional in mosses, increase of *BuGLP* mRNA in response to osmotic stress could depend on DRE in regulatory region of *BuGLP* gene. On the other hand, down-regulation of *BuGLP* by ABA suggests that the absence of ABRE in *BuGLP* regulatory region and the presence of unknown *cis*-elements that repress the transcription in response to ABA. Furthermore, post-transcriptional regulation might be involved in the control of *BuGLP*. ABA signaling of higher plants is modulated by post-transcriptional regulations mediated by RNA-binding proteins (Kuhn and Schroeder, 2003). It is possible that *BuGLP* mRNA may be destabilized by binding of RNA-binding proteins responding to ABA stimuli, resulting in the down-regulation of *BuGLP* mRNA. Although salt stress induces ROS production (Bellaire et al., 2000), the effects of salt stress and H_2O_2 on the expression of *BuGLP* is different. This may reflect that the amount of NaCl-induced ROS production is only low level compared to the addition of MV or H_2O_2 (Bellaire et al., 2000; Pastori and Foyer, 2000) and the predominance of NaCl on the regulation of *BuGLP* (Fig. 7). However, physiological significances of opposite regulation of *BuGLP* expression by ABA and H_2O_2, and osmotic stress are unclear at present.

BuGLP mRNA is abundant in cells during logarithmic phase of growth (Fig. 1). Wheat germin and a GLP (AlGLP) were also isolated from callus in which cells are

actively proliferating (Caliskan and Cuming, 1998; Neutelings et al., 1998; Tabuchi et al., 2003; Caliskan et al., 2004). These may imply that involvement of these germin and GLP in cell propagation. ABA treatment prevents DNA replication and from entering S phase in tobacco BY-2 cells, indicating that cell cycle is inhibited by ABA (Swiatek et al., 2002; Dewitte and Murray, 2003). ABA also interrupts dedifferentiation of tobacco guard cell protoplasts by blocking from entering S phase (Gushwa et al., 2003). In addition, growth of bryophytes was repressed by ABA treatment (Chopra and Mehta, 1986; Kumra and Chopra, 1986). Suppression of *BuGLP* transcription by ABA during logarithmic phase may result from inhibition of cell growth. AlGLP, which has Mn-SOD activity, highly expresses in callus but is repressed by the addition of ABA (Tabuchi et al., 2003). These suggest relation of these GLPs to cell propagation.

I examined the effect of salt stress on the expression of BuGLP. *BuGLP* mRNA was induced under salt- and osmotic-stressed condition during logarithmic phase, while salt stress did not affect *BuGLP* mRNA during stationary phase (Fig. 5). Dissociation of BuGLP from cell wall was induced by the addition of NaCl and mannitol (Figs. 3, 4 and 5). The dissociation of BuGLP from cell wall seems to be accompanied by induction of *BuGLP* expression. However, salt stress failed the induction of *BuGLP* while it promotes detachment of BuGLP during stationary phase (Fig. 5). Thus, the induction of *BuGLP* expression does not depend on the dissociation of BuGLP from cell wall. The addition of NaCl and mannitol independently induced *BuGLP* expression at the same level (Figs, 3 and 4), suggesting that the induction of BuGLP is caused by osmotic stress. Although increase of osmotic potential of medium by the addition of NaCl (200 mM) or mannitol (400 mM) is the same, the effect of non-ionic osmotic

stress caused by mannitol on the dissociation of BuGLP from cell wall is moderate compared to that of NaCl (Figs. 3 and 4). Therefore, dissociation of BuGLP caused by the addition of NaCl is provoked by increase of both osmotic stress and ionic strength of medium.

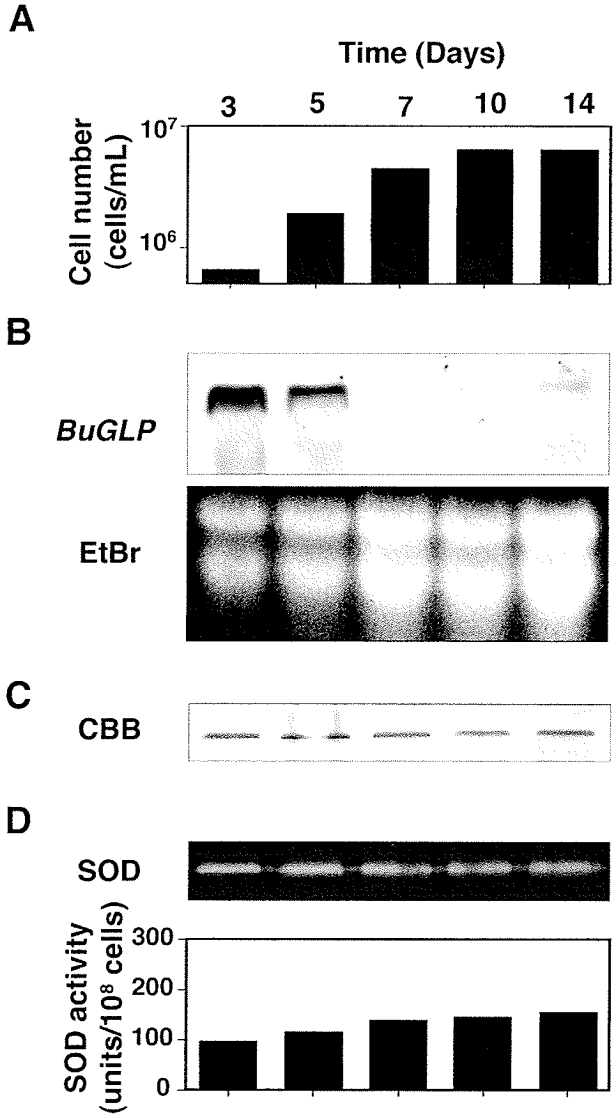

Fig. 1 *BuGLP* mRNA levels and accumulation of SOD activity of BuGLP during cell growth. Cells were grown for 3, 5, 7, 10, and 14 days and cell numbers, *BuGLP* mRNA levels, and the amount of BuGLP and SOD activities bound to cell wall were determined. A; Cell number per ml of medium. B; *BuGLP* mRNA levels demonstrated by Northern blotting. Ethidium bromide-stained RNA shows loading control. C; The amount of BuGLP protein bound to cell wall demonstrated by CBB staining after nondenaturing SDS-PAGE. The amount of protein loaded on each lane corresponds to that of 2×10^6 cells. D; SOD activity of BuGLP. Upper panel is SOD activity staining after nondenaturing SDS-PAGE carried out as C. Lower panel is SOD activities detected by SOD asssay.

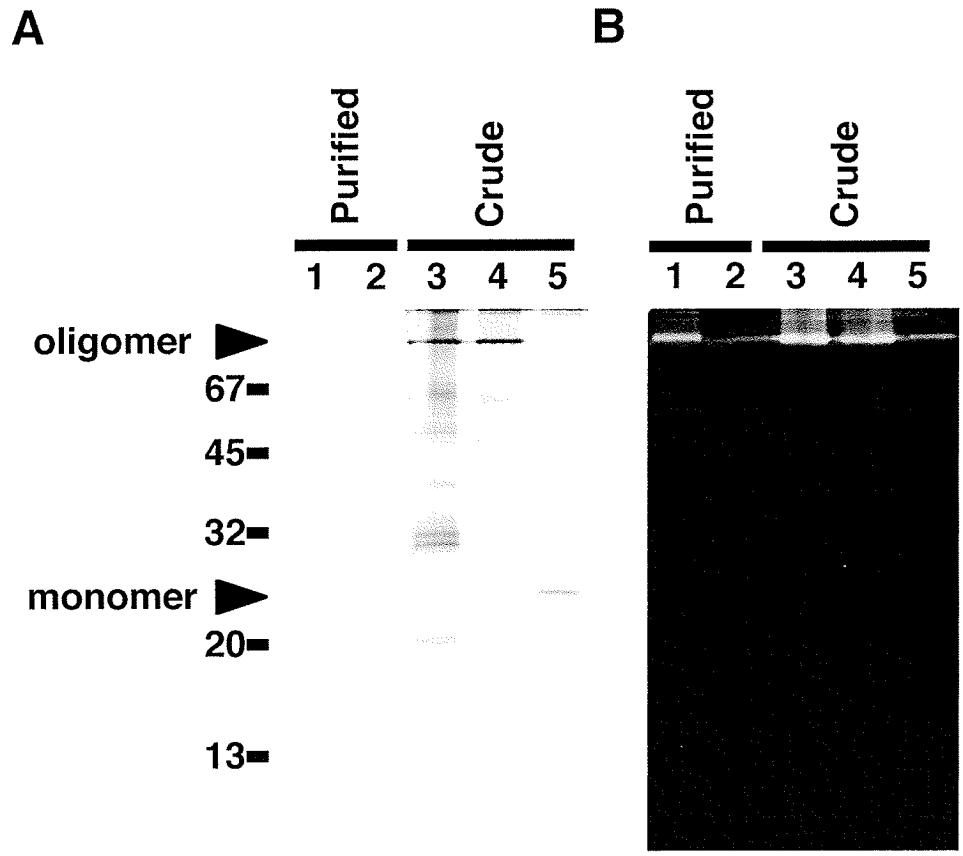

Fig. 2 Resistance of BuGLP to SDS and heat. A; CBB staining. B; SOD activity staining. Lane 1 shows purified BuGLP (5 μg per lane) as an untreated control. Upper and lower arrowheads indicate oligomeric and monomeric forms of BuGLP, respectively. Purified BuGLP (5 μg per lane) was boiled for 5 minutes in the absence of 2-mercaptoethanol and subjected to SDS-PAGE (lane 2). Lane 3 is crude extracellular protein fraction 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 minutes and subjected to SDS-PAGE as in lane 2. Molecular weights of standards are indicated on the left in kDa.

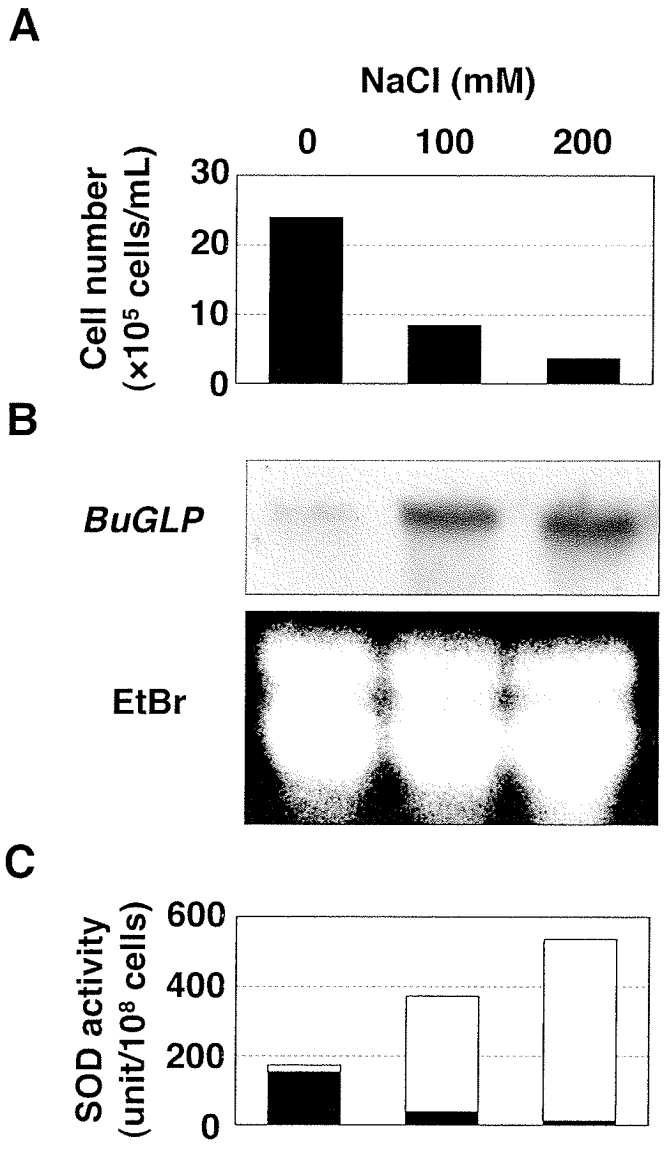

Fig. 3 Effects of salt stress on *BuGLP* mRNA levels and SOD activities of BuGLP. Cells were grown in the presence of 0, 100 and 200 mM NaCl for 5 days and cell numbers, *BuGLP* mRNA levels and SOD activities were determined. A; Cell number per ml of medium. B; *BuGLP* mRNA levels demonstrated by Northern blotting. Ethidium bromide-stained RNA shows loading control. C; SOD activity of BuGLP. Closed and opened bars indicate activities of SOD bound to cell wall and SOD released into the medium, respectively. The SOD activities are demonstrated by units per 10^8 cells.

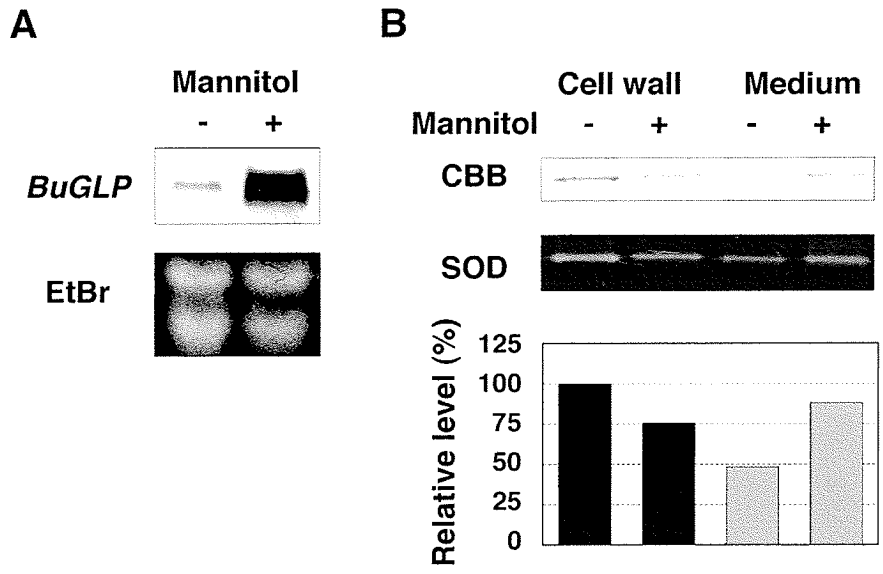

Fig. 4 Effect of mannitol on the expression of *BuGLP*. Cells were cultured for 5 days in 400 mM mannitol containing medium. RNA and cell wall-bound and medium-eluted BuGLP were extracted. A; *BuGLP* mRNA levels demonstrated by Northern blotting and ethidium bromide-stained loading control. B; the amount and SOD activities of cell wall-bound and medium-eluted BuGLP. Upper two panels show the amount of BuGLP protein demonstrated by CBB staining and SOD activity of BuGLP demonstrated by SOD activity staining, respectively. Lower panel indicates relative level of quantified SOD activity of upper panel. Black and gray bars indicate cell wall-bound and medium-eluted SOD activities of BuGLP, respectively. The levels of SOD activity of cell wall-bound BuGLP without the addition of mannitol corresponded to 100%.

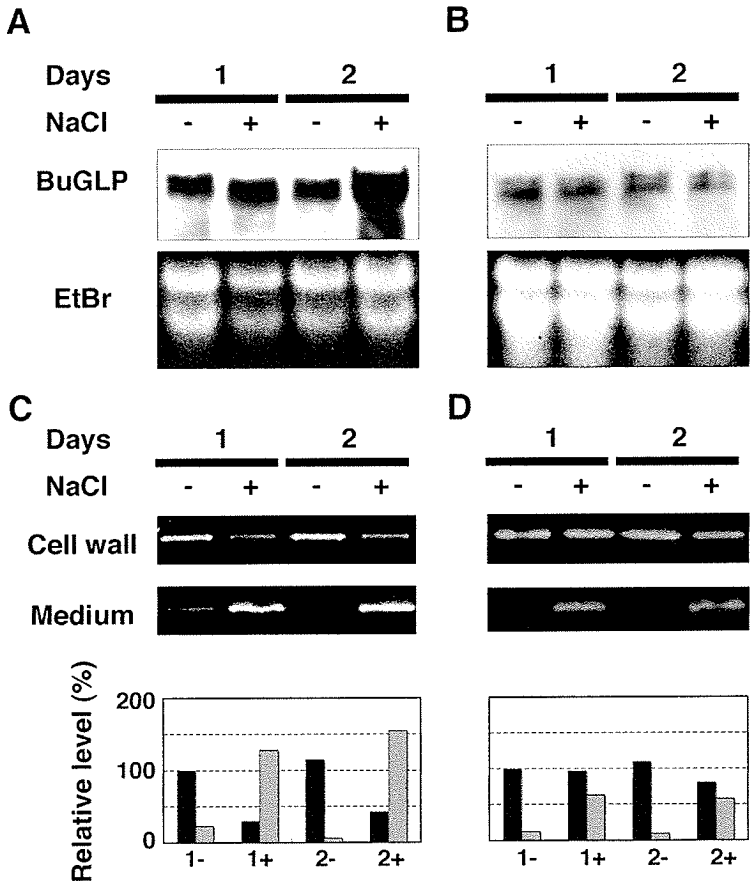

Fig. 5 Effects of salt stress on BuGLP expression in cells during the logarithmic phase and the stationary phase of growth. NaCl was added to cells cultured for 5 or 10 days to a final concentration of 200 mM. *BuGLP* mRNA levels and SOD activities bound to cell wall and released into the medium were determined at 1 and 2 days after the addition of NaCl. A and C; Cells during the logarithmic phase. B and D; Cells during the stationary phase. A and B; *BuGLP* mRNA levels were demonstrated by Northern blotting and ethidium bromide-stained loading control. C and D; upper two panels indicate SOD activities of cell wall-bound and medium-eluted BuGLP demonstrated by activity staining after nondenaturing SDS-PAGE, respectively. Lower panel indicates the relative levels of SOD activity in upper panels. Black and gray bars indicate cell wall-bound and medium-eluted SOD activities of BuGLP, respectively. The levels of SOD activity in control lane of 1 day after the addition corresponded to 100%.

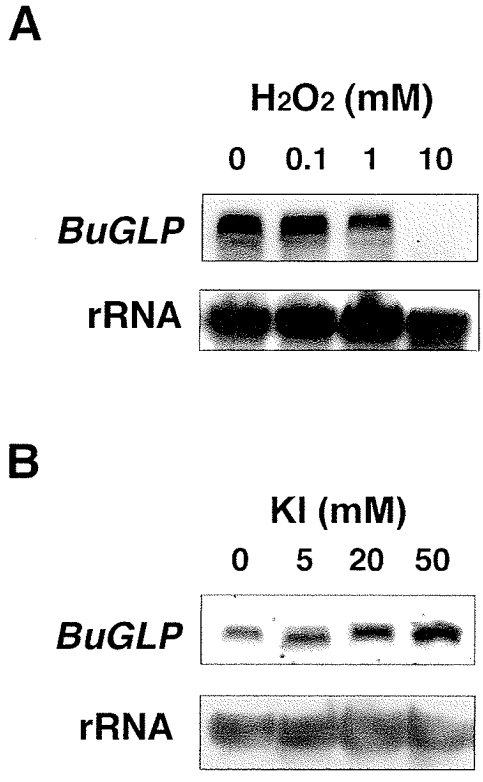

Fig. 6 Effect of H_2O_2 on the expression of *BuGLP*. H_2O_2 (A) or KI (B) was added to cells cultured for 5 days and total RNA was extracted 1 day after the addition. *BuGLP* mRNA levels demonstrated by Northern blotting and the amount of 26S rRNA was shown as loading control.

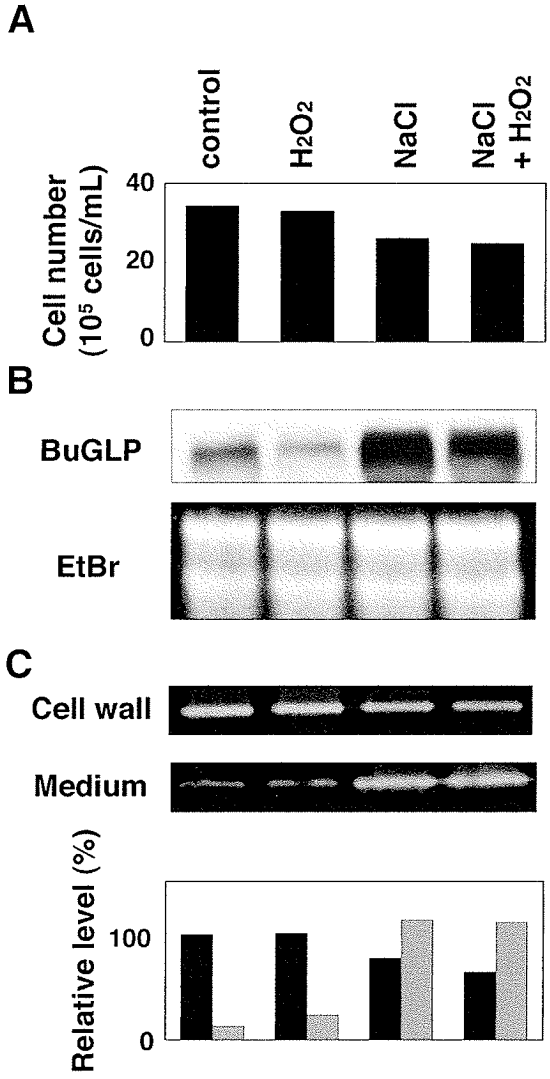

Fig. 7 Effect of NaCl and H_2O_2 on the expression of BuGLP. NaCl and H_2O_2 were added to cells cultured for 5 days to a final concentration of 200 mM and 10 mM, respectively. *BuGLP* mRNA levels and SOD activities bound to cell wall and released into the medium were determined at 1 day after the addition. A; Cell number per ml of medium. B; *BuGLP* mRNA levels demonstrated by Northern blotting and ethidium bromide-stained loading control. C; Upper two panels indicate SOD activities of cell wall-bound and medium-eluted BuGLP demonstrated by activity staining, respectively. Lower panel indicates the relative levels of SOD activity in upper two panels. Black and gray bars indicate SOD activity of cell wall-bound and medium-eluted BuGLP in upper two panels, respectively. The levels of SOD activity bound to cell wall in control lane corresponded to 100%.

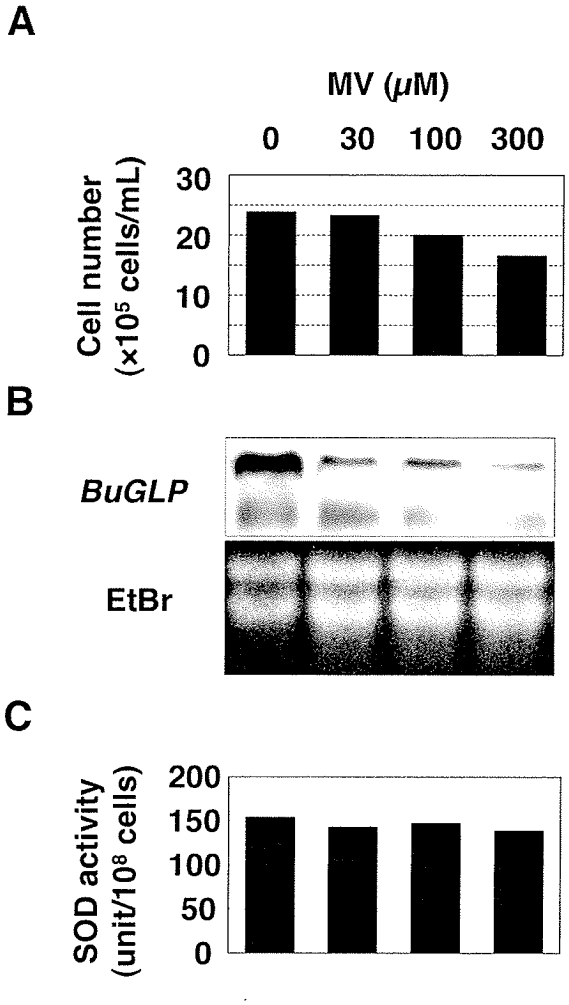

Fig. 8 Effect of MV on the expression of BuGLP. Cells were cultured in the presence of 0, 30, 100, and 300 μM MV for 5 days and cell numbers, *BuGLP* mRNA levels, and cell wall-bound SOD activities were determined. A; Cell number per ml of medium. B; *BuGLP* mRNA levels demonstrated by Northern blotting. Ethidium-bromide stained RNA shows loading control. C; Activities of SOD bound to cell wall was detected by SOD assay.

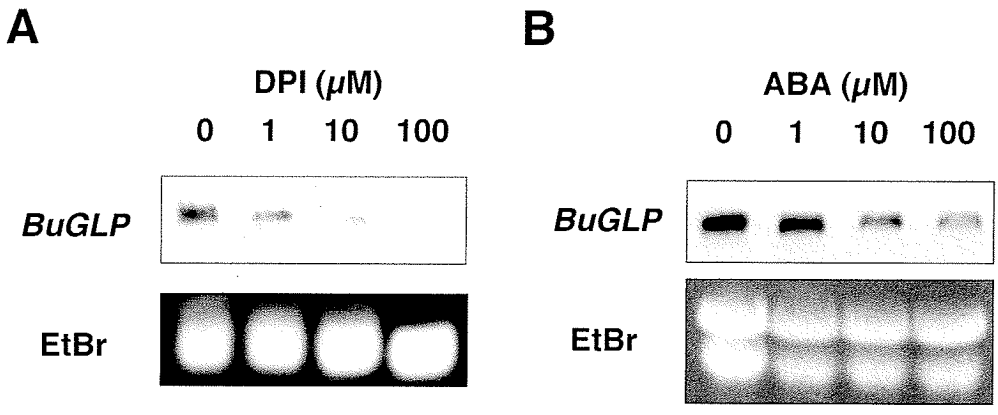

Fig. 9 Effect of DPI and ABA in the expression of *BuGLP*. DPI (A) or ABA (B) were added to 5 days cultured cells and cultured for one more day. Cells were collected and total RNA was extracted. *BuGLP* mRNA levels were demonstrated by Northern blotting and ethidium bromide-stained RNA was shown as loading control.

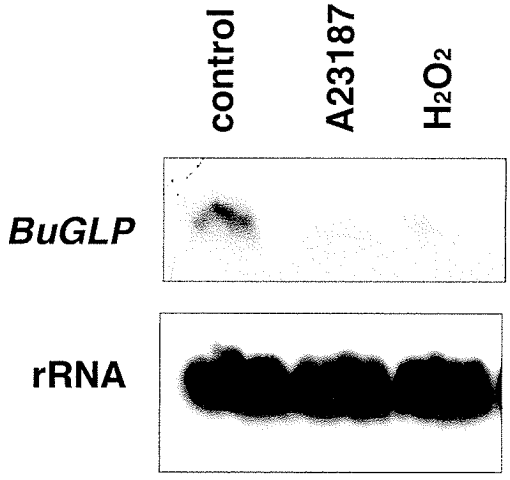

Fig. 10 Effect of increase of cytosolic Ca^{2+} concentration on the expression of *BuGLP*. A23187 (50 μM) or H_2O_2 (10 mM) was added to 5 days cultured cells and collected 1 day after the addition. *BuGLP* levels were demonstrated by Northern blotting and 26S rRNA was shown as loading control.

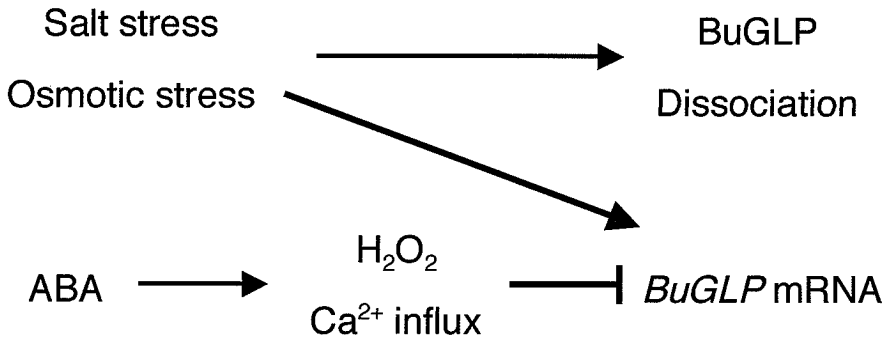

Fig. 11 A model for the effects of stimuli on the amount of *BuGLP* transcript. Osmotic stress and ABA may affect on the expression of *BuGLP* through independent signaling pathway.

CHAPTER II

Germin-like protein gene family of a moss, *Physcomitrella patens*, phylogenetically falls into two characteristic new clades

Abstract

I identified 77 expressed sequence tag (EST) clones encoding germin-like proteins (GLPs) from a moss, *Physcomitrella patens* in a database search. These *P. patens GLPs* (*PpGLPs*) were separated into 7 groups based on DNA sequence homology. Phylogenetic analysis showed that these groups were divided into two novel clades clearly distinguishable from higher plant germins and GLPs, named bryophyte subfamily 1 and 2. PpGLPs belonging to bryophyte subfamily 1 lacked two cysteines at the conserved positions observed in higher plant germins or GLPs. PpGLPs belonging to bryophyte subfamily 2 contained two cysteines as observed in higher plant germins and GLPs. In bryophyte subfamily 1, 12 amino acids, in which one of two cysteines is included, were deleted between boxes A and B. Further, I determined the genomic structure of all of 7 *PpGLP* genes. The sequences of *PpGLP*s of bryophyte subfamily 1 contained one or two introns, whereas those of bryophyte subfamily 2 contained no introns. Other GLPs from bryophytes, a liverwort GLP from *Marchantia polymorpha*, and two moss GLPs from *Barbula unguiculata* and *Ceratodon purpureus* also fell into bryophyte subfamily 1 and bryophyte subfamily 2, respectively. No higher plant germins and GLPs were grouped into the bryophyte subfamilies 1 and 2 in this analysis. Reverse transcription-PCR analysis showed that the expression of *PpGLP*s was regulated by plant hormones. Moreover, I revealed that PpGLP6 had manganese-containing extracellular superoxide dismutase activity. These results indicated that bryophyte possess characteristic GLPs, which phylogenetically are clearly distinguishable from higher plant GLPs.

Introduction

Germin and germin-like proteins (GLPs) widely distributed among land plants. Germin and GLPs are composed of three highly conserved oligopeptides that are called germin boxes A, B, and C (Bernier and Berna, 2001). The region from boxes B to C, which is called the cupin domain, contains three histidines and a glutamate involved in metal binding and forms the core beta-barrel structure. Two cysteines that stabilize the N-terminal structure by forming a disulfide bond and an N-glycosylation site (NXS/T) are conserved near box A and between box A and B, respectively. GLPs also have such biochemical properties of germin, although few of them have oxalate oxidase (OXO) activity.

Previously we isolated a GLP with manganese-containing superoxide dismutase (Mn-SOD) activity from cultured cells of a moss, *Barbula unguiculata*, and named it BuGLP (Yamahara et al., 1999). In addition to BuGLP, barley germin (Woo et al., 2000) and some GLPs such as tobacco Nectarin I are reported to have SOD activity (Carter and Thornburg, 2000; Segarra et al., 2003; Tabuchi et al., 2003). It is interesting in terms of the physiological role of GLPs that both SOD and OXO generate hydrogen peroxide (H_2O_2) and that germin and GLPs are located on the apoplast. I have examined the role of BuGLP showing that *BuGLP* mRNA is abundant during the logarithmic phase of growth, suggesting it participates cell propagation (Nakata et al., 2002). This is supported by an analysis of the promoter activity of a pine GLP using tobacco BY-2 cell (Mathieu et al., 2003). The importance of reactive oxygen species (ROS) for plant morphogenesis was also reported (Schopfer et al., 2002; Foreman et al., 2003; Mittler et al., 2004)

A large number of *GLP* genes have been discovered by expressed sequence tags (ESTs) or genomic sequencing in higher plants. The *Arabidopsis thaliana* genome contains 27 GLP genes (Carter and Thornburg, 1999; Carter et al., 1998). In barley, at least 14 GLP genes have been identified and divided into five subfamilies according to their sequence similarity (Druka et al. 2002, Wu et al. 2000). In rice, at least 8 different GLP genes are expressed (Membré and Bernier 1998). In lower plants, three *GLP* genes have been found in bryophytes, two of which were isolated from mosses, *B. unguiculata* (Yamahara et al., 1999) and *Ceratodon purpureus* (Quatrano et al., 1999), and one from a liverwort, *Marchantia polymorpha* (Nagai et al., 1999). These findings indicate that GLPs are ubiquitously distributed among land plants and constitute a large and functionally diverse gene family.

Many proteins containing cupin motifs in their amino acid sequence, which belong to the cupin superfamily, have been isolated from bacteria, archaea, and eukaryotes (Dunwell et al., 2004). The name of 'cupin' derives from the basis of a conserved beta-barrel structure ('cupa' is the Latin term for a small barrel). Among the cupin superfamily germin and the GLP family have been identified only in land plants. Although the physiological roles of germins and GLPs have not been clarified, they have stimulated interest in the evolution and diversification of their family, because all land plants appear to possess them. Bryophytes are believed to have been the first land plant group (Kenrick and Crane, 1997; Qiu and Palmer, 1999). Thus, bryophytes are considered to occupy an important position in any discussion of the evolution and diversity of GLP families. So far, an extensive phylogenetic analysis of the GLP family in bryophytes has not been reported at all. Further, a moss, *Physcomitrella patens* subsp. *patens* has been the only land plant found to have a high rate of

homologous recombination in its genomic DNA (Schaefer and Zrÿd, 1997; Schaefer and Zrÿd., 2001; Schaefer, 2002). It has a relatively small genome and a relatively simple developmental pattern (Schaefer, 2002). In addition, the main growth phase of *P. patens* is the haploid gametophyte, although that of higher plants is diploid sporophyte. Further, numerous expressed sequence tag (EST) clones have been deposited in public database (Rensing et al., 2002). These greatly helpful features should make it possible not only to clarify the physiological role of each GLP using genetic manipulations but also to study the complex gene family of *GLPs*.

In this chapter, I performed a phylogenetic analysis of the GLP family of *P. patens*, proposing two novel clades designated bryophyte subfamily 1 and 2, which are clearly distinguishable from the clades of higher plants (Carter and Thornburg, 2000). One of the most remarkable features of bryophyte subfamily 1 is a lack two cysteines at conserved positions in all the germin and GLP reported. In addition, I examined plant hormone induced expression of *PpGLP*s and identified PpGLP6 as having Mn-SOD activity.

Materials and Methods

Plant material and culture conditions

Physcomitrella patens subsp. *patens* (Ashton and Cove, 1977) was cultured at 25°C under continuous light on BCDATG agar medium (Nishiyama et al., 2000). For vegetative propagation, the plants were ground with a pestle and mortar in sterile water and soaked on the BCDATG agar medium overlaid with a layer of cellophane. For

large-scale culture of *P. patens*, the plants were ground and soaked in 2 liters of the BCDATG liquid medium. Air enriched with 1% carbon dioxide was supplied at 2 liters per minute providing the mixing force and the carbon source for growth. For investigation of the effect of plant hormones, each of plant hormones was added to BCDATG agar medium up to 1 μM.

Identification and classification of PpGLP *genes*

Sequence information on *P. patens* EST clones was obtained by searches of the *P. patens* EST database (PHYSCObase, http://moss.nibb.ac.jp/) (Nishiyama et al., 2003) and GenBank. Seven representative EST clones of each PpGLP group submitted in PHYSCObase (PpGLP1a, pphn4j19; PpGLP2, pphn38b21; PpGLP3a, pphb1d21; PpGLP4, pph28i20; PpGLP5, pphn12p01; PpGLP6, pph26d14; PpGLP7, pphb24g23) were kindly provided by Dr. Mitsuyasu Hasebe and Dr. Tomomichi Fujita (National Institute of Basic Biology, Okazaki, Japan). DNA sequencing was performed using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) and with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The localization and cleavage site of protein sorting signals of PpGLP were predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/).

Preparation of PpGLP-*specific probes*

The *PpGLP*-specific probes used for hybridization were labeled with digoxigenin (DIG) using a DIG DNA Labeling and Detection Kit (Roche diagnostics, Germany) by PCR. Template DNA fragments for DIG-labeling PCR were prepared by PCR using EST clones containing each *PpGLP* cDNA as template DNA and the *PpGLP*-specific

primers shown in Table 1. These primer sets were also used for DIG-labeling PCR.

Genomic Southern blot hybridization

Genomic DNA was extracted from *P. patens* by the CTAB method (Murray and Thompson, 1980). Ten micrograms of genomic DNA digested with *Eco*RI or *Hin*dIII was separated on a 1% agarose gel, and transferred onto nylon membrane (BIODYNE A, PALL) using 20 × SSC. Hybridization and detection were performed according to the manufacturer's instructions. The membrane was hybridized with a DIG-labeled *PpGLP*-specific DNA probe at 42°C and washed twice in 2 × SSC, 0.1% SDS for 5 minutes at room temperature and twice in 0.1 × SSC, 0.1% SDS for 15 minutes at 60°C. Hybridization signals were detected with Anti-DIG-AP conjugate and CDP-Star (Roche diagnostics).

Cloning of genomic sequences of PpGLP *genes*

The *P. patens* genomic library was provided by Dr Stavros Bashiardes as part of The "*Physcomitrella* EST Programme (PEP)" at the University of Leeds (UK) and Washington University in St Louis (USA). Manipulation of the genomic library was conducted according to standard procedures (Sambrook and Russell, 2001). Hybridization and detection were conducted as described for Southern hybridization. Phage DNA from positive plaques was digested with appropriate restriction enzymes and subcloned into plasmid vectors.

For cloning of the *PpGLP* genomic sequences by PCR, primer sets shown in Table 1 were used. PCR products were cloned into pGEM-T easy vector (Promega, WI).

Phylogenetic analysis

The deduced amino acid sequences were aligned with other known GLPs using ClustalX with default settings (Thompson et al., 1997). Phylogenetic analyses were conducted using *MEGA* version 2.1 (Kumar et al., 2001). The trees were computed from aligned sequences using the neighbor-joining (NJ) method (Saitou and Nei, 1987). The corresponding sequences of *Physarum polycephalum* spherulin (PpSPHE1a and PpSPHE1b) were included as outgroup. One thousand bootstrapped data sets were generated to estimate the statistical significance of the branching. The DDBJ/EMBL/GenBank accession numbers of the sequences analyzed in this study are: AlGLP, AB024338; AtGLP1, U75206; AtGLP2a, U75192; AtGLP2b, X91957; AtGLP3a, U75188; AtGLP3b, U75195; AtGLP4, U75187; AtGLP5, U75198; AtGLP6, U75194; AtGLP7, AF170550; AtGLP8, U75207; AtGLP9, Z97336; AtGLP10, AL138642; AtGLP11, AF058914; BnGLP, U21743; BuGLP, AB036797; CpGLP, AW098383; GhGLP1, AF116573; HvGER1, Y14203; HvGER3, AF250934; HvGER4, X93171; HvGLP1, Y15962; LeGLP, AB012138; McGLP, M93041; MpGLP, C95673; Nectarin I, AF132671; OsGLP1, AF141880; OsGLP2, AF141879; OsGLP4, AF032974; OsGLP5, AF032975; OsGLP6, AF032976; OsGLP7, AF072694; OsGLP16, AF042489; PcGER1, AF039201; PnGLP, D45425; PpABP19, U79114; PpABP20, U81162; PpGLP1a, AB177347; PpGLP1b, AB177645; PpGLP1c, AW561602; PpGLP2, AB177348; PpGLP3a, AB177349; PpGLP3b, AB177646; PpGLP4, AB177350; PpGLP5, AB177351; PpGLP6, AB177352; PpGLP7, AB177353; PpSPHE1a, M18428; PpSPHE1b, M18429; PrGLP, AF049065; PsGER1, AJ250832; PvGLP1, AJ276491; SaGLP, X84786; StGLP, AF067731; TaGER2.8, M63223;

TaGER3.8, M63224; TaGLP1, Y09915; TaGLP2a, AJ237942; TaGLP2b, AJ237943; TaGLP3, Y09917.

Since a probable frameshift was observed in the sequence data for MpGLP submitted to GenBank, a manual correction was made to obtain the most likely amino acid sequence as GLPs and the amino acid of the corrected position was expressed by X.

Purification of PpGLP with Mn-SOD activity

Twenty grams of cells of *P. patens* cultured in liquid medium for two weeks were washed using 20 mM Tris-HCl, pH 8.0, and ground with liquid nitrogen. An equal volume (w/v) of extraction buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl) was added to the frozen powder and thawed on ice. The suspension was centrifuged at 2000 × *g* for 5 min at 4°C and the supernatant was recentrifuged at 12000 × *g* for 10 min at 4°C. The supernatant was placed in a 60°C water bath for 30 min, centrifuged at 136000 × *g* for 2 hour at 4°C, and applied to a column (1 × 5 cm) of concanavalin A-agarose (Honen Co.) equilibrated with 20 mM Tris-HCl, pH 8.0. The column was washed with 15 mL of the buffer, and then the absorbed proteins were eluted with 50 mL of the buffer containing 0.5 M α-methyl-D-glucoside. The eluted proteins were treated with trypsin (10 μg/mL) for 1 hour at 37°C. To avoid excess digestion, phenylmethylsulfonyl fluoride (PMSF) (0.5 mM) was added to the trypsin-treated solution. The protein solution was concentrated with a Centriflo CF25 Membrane Cone (Amicon) and a Centricon YM-30 (MILLIPORE).

Nondenaturing SDS-PAGE and SOD activity staining were performed as described by Nakata et al. (2002). By treatment of the gels with potassium cyanide and hydrogen peroxide before SOD activity staining, the purified SOD was confirmed to be a

Mn-containing enzyme as described by Kanematsu and Asada (1989). For the analysis of the internal peptide sequence, the protein band corresponding to Mn-SOD was excised from the Coomassie brilliant blue (CBB)-stained gel and subjected to peptide mass fingerprinting.

Reverse transcription-PCR Analysis

Total RNA was extracted by modified phenol/SDS method (Chirgwin et al., 1979) and purified by RNeasy plant mini kit (Qiagen). Reverse transcription was performed by SuperScript II reverse transcriptase (Invitrogen) using an oligo dT primer. PCR was performed with first-strand cDNA for 13 or 15 cycles at 95°C for 20 sec, 50°C for 20 sec, and 72°C for 30 sec. The *PpGLP*-specific primers shown in Table 1 were used for amplification of each *PpGLP* cDNA. The products were detected by Southern blot hybridization as described above. α-tubulin was used for internal control. Effects of contamination of genomic DNA were investigated by the same analysis using total RNA as a template of PCR.

Results

Identification of PpGLP *genes from* P. patens *EST library*

To identify *P. patens GLP* (*PpGLP*) cDNA, I searched PHYSCObase that contains more than 50,000 EST clones. The EST library of PHYSCObase has been prepared from full-length enriched cDNA libraries from auxin-treated, cytokinin-treated, and untreated gametophytes of *P. patens* (Nishiyama et al., 2003). The GenBank

DNA database was also searched for the isolation of EST clones encoding *PpGLP* isolated from a cDNA library from abscisic acid-treated gametophytes. Table 2 shows 77 EST clones encoding *PpGLP* identified to date. From an alignment of the DNA sequences, they were organized into 7 groups named *PpGLP1*, *PpGLP2*, *PpGLP3*, *PpGLP4*, *PpGLP5*, *PpGLP6*, and *PpGLP7*. *PpGLP1* contained three closely related *GLP* sequences designated *PpGLP1a*, *PpGLP1b*, and *PpGLP1c*. *PpGLP3* also contained two closely related *GLP* sequences, designated *PpGLP3a* and *PpGLP3b*.

Table 3 shows the number of EST clones included in each of four cDNA libraries. The cDNA library from untreated gametophytes contains 26 clones. Auxin and cytokinin-treated libraries contain 43 and 7 EST clones, respectively. The most frequently isolated clones of *PpGLP*s are *PpGLP5* (21/77) and *PpGLP4* (19/77), both of which account for about one forth of the total number of clones encoding *PpGLP*. In contrast, a clone encoding *PpGLP6* is present only in the untreated library. The bias in the number of each *PpGLP* clones may reflect the abundance of the transcript (see below). The EST clones encoding *PpGLP5* are observed only in the auxin-treated library, and those encoding *PpGLP7* are observed only in the cytokinin-treated library. This suggests that *PpGLP5* and *PpGLP7* are induced by auxin and cytokinin, respectively. This is consistent with reports that the expression of *germin* is induced by auxin treatment (Berna and Bernier, 1999; Hurkman and Tanaka, 1996) and that the transcription of the pine *GLP* gene introduced into tobacco cell is increased by the addition of auxin and cytokinin (Mathieu et al., 2003).

Characterization of PpGLP

Representative EST clones of each of 7 *PpGLP* groups except for *PpGLP*s *1b*, *1c*

and *3b* were completely sequenced. The length of *PpGLP* cDNAs ranged from 789 to 1399 base pairs (bp). All of these full-length cDNAs consisted of a 5'-untranslated region, coding region, and 3'-untranslated region, which ranged from 68 to 435 bp, 612 to 675 bp, and 82 to 379 bp in length, respectively. The deduced amino acid sequences of each PpGLP consisted of 203 to 224 amino acid residues. Figure 12 shows the sequence identity between each PpGLP, ranging from 32.1 to 80.6 %. An analysis of PpGLP amino acid sequences using TargetP (Nielsen et al., 1997; Emanuelsson et al., 2000) revealed that there were putative extracellular targeting transit peptides composed of 18 to 29 amino acids (Fig. 13). The probability of an extracellular localization calculated by the analysis was more than 93 % for every PpGLP except PpGLP4 (86 %).

All of the PpGLPs shared three highly conserved domains called germin boxes A, B, and C. Boxes B and C of all PpGLPs contained the three histidines and the glutamate residues involved in metal binding. This suggests the presence of a manganese ion in PpGLPs as described in other GLPs (Requena and Bornemann, 1999; Yamahara et al., 1999; Carter and Thornburg, 2000; Woo et al., 2000)

The lengths of the entire amino acid sequences of PpGLPs were relatively flexible. The putative mature PpGLPs 2, 4, 5, and 7 were 182 to 186 amino acids long. I found that 12 amino acids were deleted between boxes A and B in these PpGLPs, and named them short PpGLPs (Fig. 13). On the other hand, the putative mature PpGLPs 1, 3, and 6 were 194 to 202 amino acids long, and I named them long PpGLPs. The sequence identity among short PpGLPs was more than 66.7 % and among long PpGLPs ranged from 43.3 to 54.5 % (Fig. 12).

In higher plants, most known germins and GLPs have two conserved cysteines,

which form a disulfide bond to stabilize its structure, one located in box A and the other between boxes A and B (Bernier and Berna, 2001; Woo et al., 2000). In *P. patens*, however, the short PpGLPs, PpGLPs 2, 4, 5, and 7, contained no cysteines; the former cysteine residue is substituted with tyrosine or phenylalanine and the latter situates in the deleted region. Further, PpGLPs 2 and 7 contained a single and unpaired cysteine located at an unfixed position. This is partly consistent with a report that some GLPs of *Arabidopsis thaliana* (AtGLP) contain a single cysteine in addition to paired-cysteines (Carter et al., 1998).

Possible N-glycosylation sites are also found in most germins and GLPs at a fixed position between boxes A and B (Bernier and Berna, 2001). Although PpGLPs 3, 4, 6, and 7 had a single putative N-glycosylation site as found in GLPs from higher plants, these sites were located at unfixed positions; for PpGLP3a and 3b near the middle of the protein (N138), PpGLP4 in germin box A (N33), PpGLP6 near the C-terminus (N190), and PpGLP7 near germin box A (N40).

An additional important feature observed in more than half of all GLPs reported is the presence of RGD-like tripeptides, which interact with cell adhesion proteins and integrins in animals (Ruoslahti and Pierschbacher, 1987). The discovery of RGD tripeptides in GLPs has led to the suggestion that the proteins participate in interaction with other extracellular proteins as receptors (Swart et al., 1994). PpGLPs 1, 6 and 7 also had a putative RGD-like tripeptide and PpGLP6 had two RGD-like tripeptides.

Phylogenetic analysis of PpGLP

To examine phylogenetic relationships among germins and GLPs, Carter and Thornburg (1999; 2000) used the whole protein sequences of germins and GLPs as

queries and identified five separate clades, whereas some groups (Khuri et al., 2001; Kim and Triplett, 2004) used the protein sequence of the cupin domain, which consists of germin boxes B and C, and an inter box region, and identified three more functionally integrated clades. In the present study, both sequences were used for phylogenetic analyses of PpGLPs and other GLPs and two dendrograms were generated (Fig. 14). Germins and GLPs used in this study include 26 dicotyledonous sequences (from *A. thaliana* and other plant), 17 monocotyledonous sequences (from wheat, barley, and rice), 2 gymnosperm sequences (from pines), and 13 bryophyte sequences including 10 sequences from *P. patens*, one each from the mosses *B. unguiculata* and *C. purpureus*, and one from a liverwort, *M. polymorpha*. In addition, two fungal spherulins (Bernier et al., 1987) were used as outgroup. Figure 14A shows a dendrogram obtained using whole amino acids sequences. As reported by Carter and Thornburg (2000), the tree showed that germins and GLPs from higher plants were classified into five clades, called the true germin subfamily, gymnosperm subfamily, and GLP subfamilies 1, 2, and 3. For example; wheat and barley germin with OXO activity and some GLPs are included in the true germin subfamily; AlGLP and Nectarin I, both of which have Mn-SOD activity (Carter and Thornburg, 2000; Tabuchi et al., 2003), are classified into subfamily 1 and 2, respectively; peach auxin-binding proteins and HvGLP1 that have ADP-glucose pyrophosphatase/phosphodiesterase (AGPPase) activity (Rodríguez-López et al., 2001) belong to subfamily 3; two pine GLPs and an *Arabidopsis* GLP (AtGLP7) were included in the gymnosperm subfamily. In addition to the five phylogenetic clades, two novel clades were generated by this analysis of 9 PpGLP sequences. All of the *P. patens* GLP sequences fell into two novel clades. They were designated bryophyte subfamilies 1 and 2, consisting of PpGLPs 2, 4, 5, and

7, and PpGLPs 1a, 1b, 3a, 3b, and 6, respectively. As to other GLPs from bryophytes, BuGLP isolated from the moss *B. unguiculata* as a protein with extracellular Mn-SOD activity (Yamahara et al., 1999) was classified into bryophyte subfamily 2 and MpGLP isolated from the liverwort *M. polymorpha* (Nagai et al., 1999) was included in bryophyte subfamily 1. Interestingly, the two novel bryophyte subfamilies contained no GLPs from higher plants.

Figure 14B shows the dendrogram obtained using the amino acid sequence of the cupin domain. In this analysis, PpGLP1c from *P. patens* and CpGLP from a moss, *C. purpureus*, were included, since only the sequences of cupin domains have been submitted to the database. The resulting phylogenetic tree has a similar topology to that shown in Fig. 14A. GLPs from higher plants formed five clades described above and bryophyte GLPs also formed two novel clades, bryophyte subfamilies 1 and 2. Long PpGLPs and other moss GLPs fell into bryophyte subfamily 2, and short PpGLPs and a liverwort GLP fell into bryophyte subfamily 1. These results enhanced the reliability of the trees shown in Fig. 14A and attested that all of the bryophyte GLPs studied are separated into two novel bryophyte subfamilies.

Khuri et al. (2001) suggested that the dendrogram obtained using the cupin domain gives a more complete picture of the functional relationships among GLPs. The result obtained in this study, however, showed that BuGLP, AlGLP and Nectarin I which have SOD activity were separated into different clades, bryophyte subfamily 2, subfamily 1, and subfamily 2, respectively, suggesting that the phylogenetic tree obtained using the cupin domain does not reflect biochemical properties of germins and GLPs.

The phylogenetic analysis in this study was carried out by the NJ method,

whereas Khuri et al. (2001) performed the analysis using the maximum-parsimony (MP) method. Then, using the MP method I also generated phylogenetic trees based on the whole amino acid sequences and the cupin domain sequences. The trees obtained by the MP method were fundamentally the same as those generated using the NJ method (data not shown).

Genomic Southern blot analysis of PpGLP*s*

To understand the copy number of each *PpGLP* gene, a genomic Southern blot analysis was performed (Fig. 15). Since the amino acid sequences of the cupin domain of PpGLPs and their nucleotide sequences are very similar to each other, probes containing entire *PpGLP* coding sequences were thought to possibly cross-hybridize to other *PpGLP* genes. To avoid such a cross-hybridization and generating incorrect signals, I designed *PpGLP* gene-specific probes using nucleotide sequences between germin boxes A and B except for the probes for *PpGLP*s *1* and *3*. *PpGLP1* probe was designed using the N-terminus region of *PpGLP1a* and *PpGLP3* probe using the 3'-untranslated region of *PpGLP3a*. To confirm the specificity of the *PpGLP* probes, each probe was hybridized with plasmid DNA containing each of the *PpGLP* cDNA. No hybridization occurred with other *PpGLP* cDNAs (data not shown). However, the probe for *PpGLP1* and for *PpGLP3* detected the related genomic sequences of *PpGLP*s *1b* and *1c*, and *PpGLP3b*, respectively (data not shown).

*Eco*RI and *Hin*dIII were used as the restriction enzymes for digestion of genomic DNA, since these enzymes do not digest DNA sequences used for the *PpGLP* probes. The *PpGLP 2*, *4*, *5*, *6*, and *7* probes detected only one signal in each lane, indicating that their genes exist as a single copy in the genome. On the other hand, the *PpGLP*s *1* and

3 probes detected two to four signals, indicating that there are more than two genes similar to *PpGLP1* and *PpGLP3* in the genome. This result was consistent with the existence of *PpGLP 1b* and *1c*, and *3b* cDNAs, respectively, obtained from the database search (Tables 2 and 3).

Cloning of genomic PpGLP *genes*

The genomic structure of the *PpGLP* genes was determined. Genomic *PpGLP* genes were first isolated by screening a *P. patens* genomic library and phage clones containing *PpGLPs 1a*, *1b*, *3b*, and *6* genes were identified. Clone number g102 hybridized with the *PpGLP1*-specific probe. The *Spe*I-digested DNA hybridized with the same probe showed two strong signals. The sequence of the double-digested (*Spe*I and *Sph*I) fragment (5 kbp) contained two ORFs of the *GLP* gene. One was identical to *PpGLP1a* cDNA and the other identical to *PpGLP1b* cDNA (Fig. 16). The amino acid identity between PpGLP1a and PpGLP1b was 97.2 %. The length of the intergenic region between the two ORFs was only 1004 bp. Similar tandem-repeated *GLP* genes were identified by a physical and genetic mapping of barley *GLPs* that contain a *GLP* gene-rich region encoding nine *GLP* genes (Druka et al., 2002). Clone number g81 hybridized with the *PpGLP3*-specific probe. The *Spe*I fragment (2.5 kbp) of g81 was subcloned and the sequence contained an ORF identical to *PpGLP3b* cDNA. The identity of the deduced amino acid sequence between PpGLP3b and PpGLP3a was 97.7 %. Clone number g114 hybridized with the *PpGLP6*-specific probe. The double-digested (*Hin*dIII and *Sal*I) fragment (2.9 kbp) of g114 was subcloned and the contained an ORF identical to *PpGLP6* cDNA. Genomic DNA sequences of *PpGLPs 1a*, *1b*, *3a*, and *6* were identical to their cDNAs, indicating that no introns exist in their

genes.

Although the isolation of other *PpGLP* clones from the library was unsuccessful, I successfully isolated other genes, *PpGLP 2*, *3a*, *4*, *5*, and *7*, by genomic PCR. The genomic sequence of *PpGLP3a* was identical to its cDNA. The genomic sequences of *PpGLP 2*, *4*, and *5* showed the existence of a single intron, the length of which was from 245 to 544 bp. In contrast, *PpGLP7* contained two introns, the lengths of which were 153 and 209 bp (Fig. 16). All of these introns were located about 10 to 20 bp upstream from the first methionine codon. Although this observation was consistent with the report that most of the *GLP* genes contained a single intron at a conserved position in higher plants (Bernier and Berna, 2001), the positions of introns in the short *PpGLP*s are different from those of higher plant *GLP*s in which an intron interrupts the coding region of the *GLP*s near the N-terminus of its mature protein. Interestingly, all of the *PpGLP* genes containing introns were short types (*PpGLP 2*, *4*, *5*, and *7*) falling into bryophyte subfamily 1, and the others were long types (*PpGLP 1a*, *1b*, *3a*, *3b*, and *6*) falling into bryophyte subfamily 2 (Fig. 14).

The effect of plant hormones for the expression of PpGLPs

Plant hormone-regulated expression patterns of some germins and GLPs have been reported (Hurkman and Tanaka, 1996; Berna and Bernier, 1999; Mathieu et al., 2003). In order to investigate the effect of plant hormones for the expression of *PpGLPs*, *P. patens* was cultured on agar medium containing plant hormones for 10 days. The amount of each *PpGLP* transcripts was quantified by RT-PCR analysis. Under normal condition all of *PpGLPs* except for *PpGLP7* were expressed (Fig. 17). The members of *PpGLP* gene family as a whole seemed to be repressed by NAA except for

PpGLP2. BA slightly increased *PpGLP4* and *PpGLP6* transcripts and decreased *PpGLP1b*, *PpGLP2*, *PpGLP3*, and *PpGLP5*. GA_3 induced *PpGLP1a*, *PpGLP3*, *PpGLP5*, and *PpGLP6* expression. ABA repressed *PpGLP1b*, *PpGLP4*, and *PpGLP6*. These results indicated that the expressions of all *PpGLP*s are regulated by plant hormones. Distribution of ESTs presented in Table 3 predicted that *PpGLP*s *2* and *5*, and *7* are induced by auxin and cytokinin, respectively. The prediction was basically consistent with my RT-PCR analysis, but the expression of *PpGLP5* in auxin treatment was not. Pp*GLP7* expressed only under cytokinin-supplemented condition, suggesting its physiological roles for the cytokinin-related phenomenon such as bud formation.

Identification of PpGLP with Mn-SOD activity

Previously we isolated a protein with extracellular Mn-SOD activity from a moss, *B. unguiculata*, and identified it as a member of the GLP family (BuGLP) (Yamahara et al., 1999). When the crude extract of cell wall protein of *P. patens* was subjected to nondenaturing SDS-PAGE followed by SOD activity staining, a protein with an apparent molecular mass of about 130 kDa was observed to have Mn-SOD activity (data not shown). Since in molecular mass it was similar to BuGLP (Yamahara et al., 1999) and the protein was extracted from intact cells into buffer containing 1 M NaCl in a similar manner as BuGLP (Nakata et al., 2002), the protein was thought to be a PpGLP. Then, the Mn-SOD was purified with reference to the biochemical properties of GLPs such as heat stability (Carter and Thornburg, 2000; Nakata et al., 2002), existence of glycan (Yamahara et al., 1999; Woo et al., 2000), and tolerance to serine protease (Lane, 1994; Membré et al, 2000; Segarra et al., 2003) as described in materials and methods. The purified protein was separated by nondenaturing

SDS-PAGE followed by CBB staining and SOD activity staining (Fig. 18). One protein band with a molecular mass of about 130 kDa was observed to have Mn-SOD activity. Internal peptide sequence analysis revealed that it contained two peptide sequences, FVTQL and NGFRTDQATVQIR, indicating that the protein was PpGLP6. PpGLP6 was closely related to BuGLP phylogenetically and fell into bryophyte subfamily 2 (Fig. 14).

Discussion

Previous phylogenetic studies on the *GLP* gene family have focused mainly on higher plant *GLPs*. Only one or two bryophyte GLP sequences were included in their analyses and bryophyte GLPs were grouped into the subfamilies of some higher plant GLPs (Neutelings et al., 1998; Carter and Thornburg, 2000; Khuri et al., 2001; Kim and Triplett, 2004). The data reported here provide the first phylogenetic characterization of GLPs of a moss, *P. patens*, and indicate that bryophytes, which are thought to be the most primitive land plants, have *GLP* gene families considerably different from those of higher plants. Analyses based on either whole amino acid sequences or the cupin domains indicated that PpGLPs were integrated into two novel clades, bryophyte subfamilies 1 and 2, which had not been identified during the studies of higher plant GLPs. In addition, other bryophyte GLPs such as those from mosses, *B. unguiculata* and *C. purpureus*, and a liverwort, *M. polymorpha*, were also grouped into the bryophyte clades. In contrast, 13 of *Arabidopsis* GLPs (AtGLPs) analyzed in this study revealed that AtGLPs were grouped into five clades other than bryophyte

subfamilies 1 and 2. Gymnosperm GLPs, however, fell into only one of the five clades, and the gymnosperm subfamily also contained one AtGLP. These results suggested that bryophyte GLPs are divided into two peculiar subfamilies, which are clearly distinguished from those of higher plants.

The most significant feature of bryophyte GLPs is the existence of short GLPs (bryophyte subfamily 1) which have a deletion of 12 amino acids between germin boxes A and B and lack two cysteine residues at the conserved positions observed in all GLPs from higher plants (Fig. 13). It has been clarified by crystallographic analysis of barley germin that these two cysteines stabilize the N-terminal extension (Woo et al., 2000). Therefore, these short PpGLPs might be susceptible to heat and protease treatment. It is interesting that the short PpGLPs were clearly distinguishable from the long PpGLPs in the phylogenetic analysis using even the cupin domain, although the amino acid sequence of the cupin domain was not relevant to the length of the entire amino acid sequence of PpGLP. Moreover, from the viewpoint of the existence of introns in the genomic structure, *PpGLP* genes were divided into short and long PpGLPs; the short ones (bryophyte subfamily 1) have introns and the long ones (bryophyte subfamily 2) have no introns (Fig. 16). In summary, the most characteristic features of bryophyte GLPs are that they can be clearly distinguished by the absence or presence of two cysteines, short or long GLPs, and presence or absence of introns.

Based on a search of the EST database and genomic Southern blot analysis, the total number of *PpGLP* genes in the genome of *P. patens* was assumed to be about 13 (Fig. 15). In *A. thaliana*, 30 GLP sequences are used for the phylogenetic analysis (Carter and Thornburg, 2000), and the total number of *GLP* genes is estimated at around

40 (Bernier and Berna, 2001). The genome sizes of *A. thaliana* and *P. patens* are 125 Mbp and 511 Mbp, respectively (Reski and Cove, 2004). Thus, numbers of *GLP* genes are not in proportion to genome size. In my analysis AtGLPs were widely distributed among all of five subfamilies of higher plant GLPs. This suggests that the *GLP* gene family diversified in higher plants during evolution.

In higher plants, GLPs are expressed in all parts of the plant and at all developmental stages. In mosses, plant hormones are also involved in the determination of morphogenesis (Reski, 1998). For example, in *P. patens*, auxins promote caulonema and rhizoid formation, and cytokinins induce bud formation (Ashton et al., 1979; Schumaker and Dietrich, 1998). RT-PCR analysis revealed that the transcript of *PpGLP7* was obviously induced by cytokinin and the amount of other *PpGLP* transcripts was affected by plant hormones (Fig. 17). Moreover, peach auxin-binding proteins are reported to be members of the GLP family (Ohmiya et al., 1998) and the crystal structure of maize auxin-binding protein 1, which belongs to the cupin superfamily, resembles the dimer form of germin (Woo et al., 2002). These findings suggest that the transcription of some *PpGLP* genes is regulated by plant hormone and that these PpGLPs are involved in plant hormone-induced reactions.

GLPs usually are localized to the cell wall, which plays important roles in the determination of shapes of cells (Bernier and Berna, 2001; Cosgrove, 2000). Both OXO and SOD found in the GLP family generate H_2O_2, a ROS which functions to cross-link cell wall components. Wheat germin restricts germinative growth via the local supply of H_2O_2 for cross-linking of cell wall components (Caliskan and Cuming, 1998) and its OXO activity generating H_2O_2 is reported not to be involved in the efficiency of penetration of a fungus *Blumeria graminis* f.sp. *tritici* into plants and

rather to play a role as a structural protein (Schweizer et al., 1999). On the other hand, ROS generated on the cell wall is required in the extension growth of maize seedlings induced by auxin (Schopfer et al., 2002), and root and root hair growth of *A. thaliana* (Foreman et al., 2003). Since reconstruction of the cell wall is an important event in plant morphogenesis regulated by plant hormones, some GLPs may play essential roles in plant morphogenesis.

Among bryophytes, liverworts are considered to be more primitive land plants than mosses (Nickrent et al., 2000; Qiu et al., 1998). MpGLP from the liverwort *M. polymorpha* contained no cysteines at conserved positions and belongs to bryophyte subfamily 1. This may suggest that short PpGLPs and MpGLP in bryophyte subfamily 1 have the biochemical features of GLPs acquired by ancestral land plants. The number of EST clones of short PpGLPs from auxin and cytokinin-treated gametophores is 37/43 and 7/7, respectively, and that from non-treated gametophores is 14/26. The predicted hormonal responses of the members of *PpGLP* gene family were confirmed by RT-PCR (Fig. 17). These results indicate that the short *PpGLP* genes are expressed at a higher rate than the long *PpGLP* genes, especially when they are grown on plant hormone-containing medium. It might be speculated that the ancestor of land plants such as the liverworts acquired GLPs resembling short PpGLPs and that long-type GLPs arose during the process of evolution and diversification of land plants.

	name	Sequence (5' to 3')
probe	PpGLP1-f	GTGGTTTTCTCTCACCCAAC
	PpGLP1-r	ACGCGAATCCAAGGGTGTTC
	PpGLP2-f	AATTGGCTCGTGTCATGTTCG
	PpGLP2-r	GGAAGGTACTTTGATCATGGC
	PpGLP3-f	CGCATGAGCAATCTAGGAGA
	PpGLP3-r	CTCAATTCCGCAGCGACAATC
	PpGLP4-f	GGCAATTCCTATAGCTCTCG
	PpGLP4-r	GGCCAGGTACTTGATCATAAGC
	PpGLP5-f	CCGTTTCGCTTTAGTGATGC
	PpGLP5-r	GGGCAAGTACTTGATCTGGG
	PpGLP6-f	TCTTCTCTACCTTGTCGTCC
	PpGLP6-r	CACATCGAAATCAAGACGCG
	PpGLP7-f	CTCTGGTGATGATGATCACC
	PpGLP7-r	GGGCAAGTATTTGATCTGGG
	tubulin-f	GAGCCATACAACAGCGTGCTGTC
	tubulin-r	GCCGTGTTGTTGCTGATCATGCAC
genome cloning	PpGLP2-5'	GAGTTTCCGTGTGCCAAGTTT
	PpGLP2-3'	CTTTGCTTCTCCTAGTCAGA
	PpGLP3a-5'	TCACTTCATTCACCAAGCACT
	PpGLP3a-3'	AAGTGTGATGACAACACTTGGT
	PpGLP4-5'	AGCAGAACATCCCACACAATTC
	PpGLP4-3'	AAAATGAGTGTGGATAGGGCTCT
	PpGLP5-5'	TGAGGAGTCATTCAGTGTTCA
	PpGLP5-3'	ATCAATCAAAGAATGCAGCTTTTATC
	PpGLP7-5'	CACCGGGCACTTGTAATACG
	PpGLP7-3'	CGGCCAATTTGCATGTATTAG

Table 1 Primers used in this study

GLP locus	GenBank accession No.	EST clone No.	GLP locus	GenBank accession No.	EST clone No
PpGLP1a	BQ041630	gd24e11.y1	PpGLP4	BJ201011	pphn41c19
	BQ041376	gd21c11.y1		BJ199389	pphn34d24
	BQ039801	gd03c11.y1		BJ192855	pphn17f08
	AW598786	ga88a05.y1		BJ186180	pphb9o06
	AW700000	gb36h10.y1		BJ176460	pphb17a22
	BI740810	gc85g09.y1		BJ170806	pph26o24
	BQ040182	gd12e08.y1		BJ160327	pph18l13
	BJ198512	pphn32b01		BJ171308	pph28i20
	BJ194675	pphn21d16		BJ171117	pph27p09
	BJ203814	pphn4j19		BJ192557	pphn16n11
PpGLP1b	BQ040536	gd17a06.y1		BJ170728	pph26k24
	AW476865	ga38c04.y1		BJ163025	pph30d10
PpGLP1c	AW561602	ga82c05.y1		BJ205583	pphn8f09
PpGLP2	AW127002	ga18g05.y1		BJ159694	pph16n11
	AW561342	ga78h10.y1	PpGLP5	BJ191116	pphn12p01
	AW477110	ga42a05.y1		BJ200119	pphn3k01
	BJ200717	pphn40k22		BJ203608	pphn49a18
	BJ196807	pphn27m03		BJ207024	pphn39g23
	BJ195830	pphn24o24		BJ206472	pphn38i05
	BJ202858	pphn47c12		BJ202045	pphn44i21
	BJ190566	pphn10l19		BJ201021	pphn41d23
	BJ194333	pphn20f16		BJ198208	pphn31h05
	BJ194821	pphn21p21		BJ197742	pphn20a1
	BJ198850	pphn33b06		BJ197459	pphn29k23
	BJ206688	pphn38b21		BJ196794	pphn27j03
	BJ607445	pphn39d14		BJ196283	pphn26j07
PpGLP3a	AW561616	ga82d09.y1		BJ195907	pphn25o03
	AW476993	ga40c03.y1		BJ195265	pphn23a10
	AW599752	ga92e09.y1		BJ193246	pphn18m09
	BQ827336	gd54f05.x1		BJ191738	pphn14b05
	BJ189259	pphb45p23		BI436831	gc53a06.y1
	BJ177519	pphb1d21		BI436464	gc47b02.y1
PpGLP3b	AW561724	ga85a08.y1		BJ196284	pphn26j08
PpGLP4	BJ163451	pph35j01		BJ203119	pphn48d05
	BJ163246	pph34m16		AW145102	ga29c07.y1
	BJ162520	pph25g14	PpGLP6	BJ170586	pph26d14
	BJ162563	pph25i19	PpGLP7	BJ179267	pphb24g23
	BJ206373	pphn37e05		BJ189273	pphb46c03
				BJ567342	pphb33n04

Table 2 List of EST clones coding PpGLP.

	PpGLP1a	PpGLP1b	PpGLP1c	PpGLP2	PpGLP3a	PpGLP3b	PpGLP4	PpGLP5	PpGLP6	PpGLP7
untreated (26)	5	2	1	3	2	1	11	0	1	0
auxin (43)	5	0	0	10	1	0	6	21	0	0
cytokinin (7)	0	0	0	0	2	0	2	0	0	3
abscisic acid (1)	0	0	0	0	1	0	0	0	0	0
total (77)	10	2	1	13	6	1	19	21	1	3

Table 3 Distribution of ESTs coding *PpGLP* among cDNA libraries from plant hormone-treated gametophytes of *Physcomitrella patens*. The numbers in parentheses indicate the total number of clones obtained from each cDNA library.

PpGLP1a	PpGLP1b	PpGLP2	PpGLP3a	PpGLP3b	PpGLP4	PpGLP5	PpGLP6	PpGLP7	
	97.2	37.5	52.1	52.1	32.1	35.0	43.3	35.8	PpGLP1a
		37.5	52.6	52.6	43.5	35.0	43.3	35.8	PpGLP1b
			41.0	41.1	67.5	72.0	38.3	66.2	PpGLP2
				97.7	39.9	33.3	54.5	34.6	PpGLP3a
					39.9	32.8	54.5	35.7	PpGLP3b
						67.8	46.6	69.0	PpGLP4
							34.0	80.6	PpGLP5
								34.7	PpGLP6
									PpGLP7

Fig. 12 Percent amino acid identity between PpGLPs. The entire amino acid sequences of PpGLPs are compared to determine the identity between each pair of sequences. Since for PpGLP1c only a partial amino acid sequence is available, it is excluded from this figure. The amino acid sequences of PpGLP1b and 3b are derived from their genomic sequences.

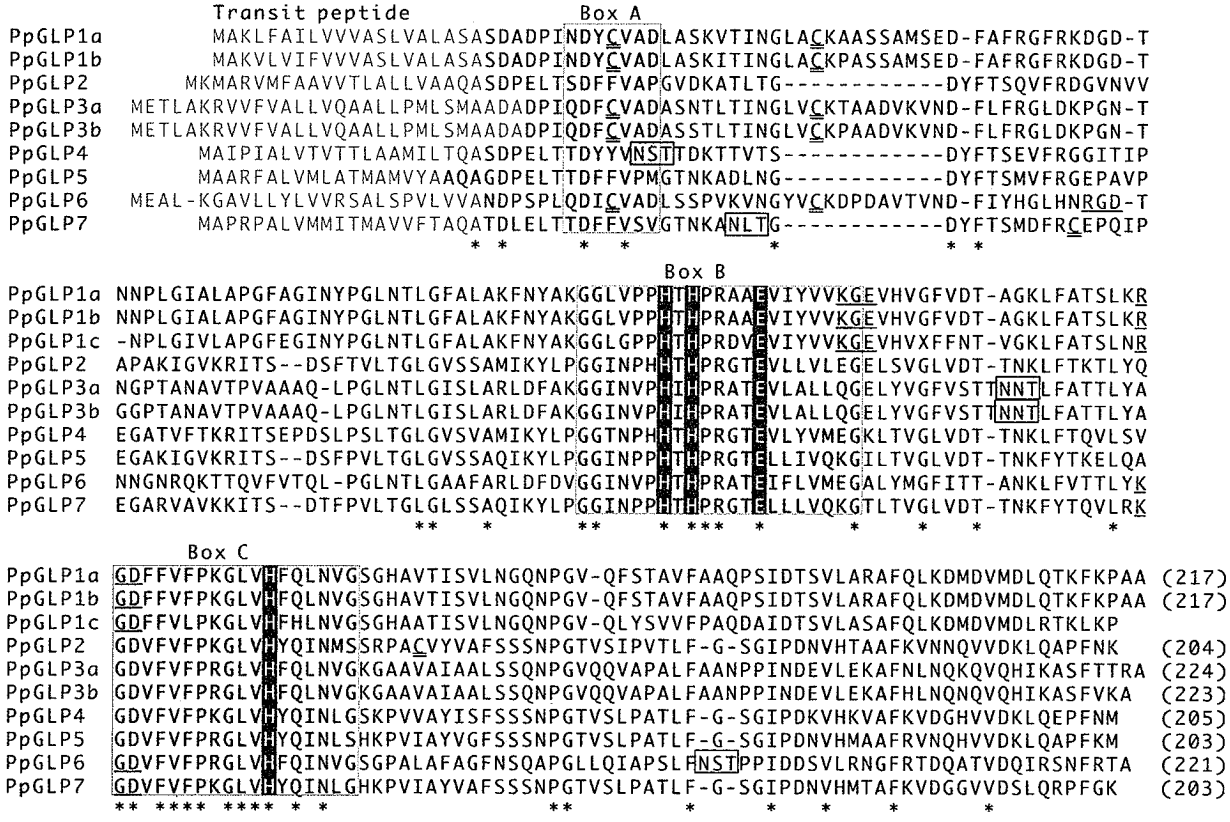

Fig. 13 Multiple alignments of amino acid sequences of PpGLPs. Ten amino acid sequences of PpGLPs are aligned. Asterisks indicate the strictly conserved residues and three conserved germin boxes are highlighted with gray. Three histidines and a glutamate that are involved in metal binding are indicated with gray boxes. Opened boxes indicate putative N-glycosylation sites. Transit peptides, the RGD-like motif, and cysteines are indicated by gray, an underline, and a double underline, respectively.

A

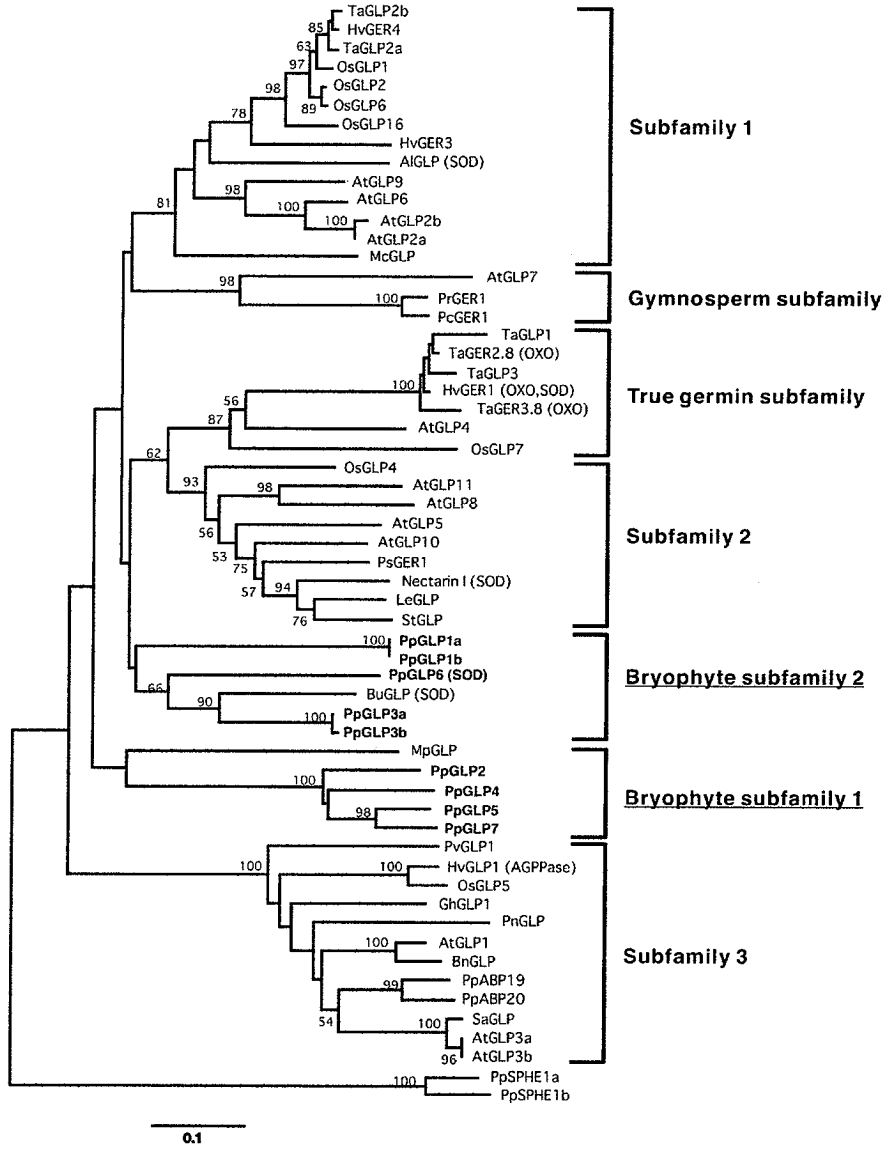

Fig. 14 Phylogenetic tree of plant GLP proteins. The tree was computed from the aligned whole amino acid sequences (A) and cupin domain (B) using the NJ method. Local bootstrap probabilities of more than 50 % are shown above or near the branch. The names of each subfamily are from Carter and Thornburg (2000). Enzymatic activities of GLPs previously reported are shown in parentheses. PpGLPs are shown in bold. Two novel clades containing PpGLPs are underlined.

B

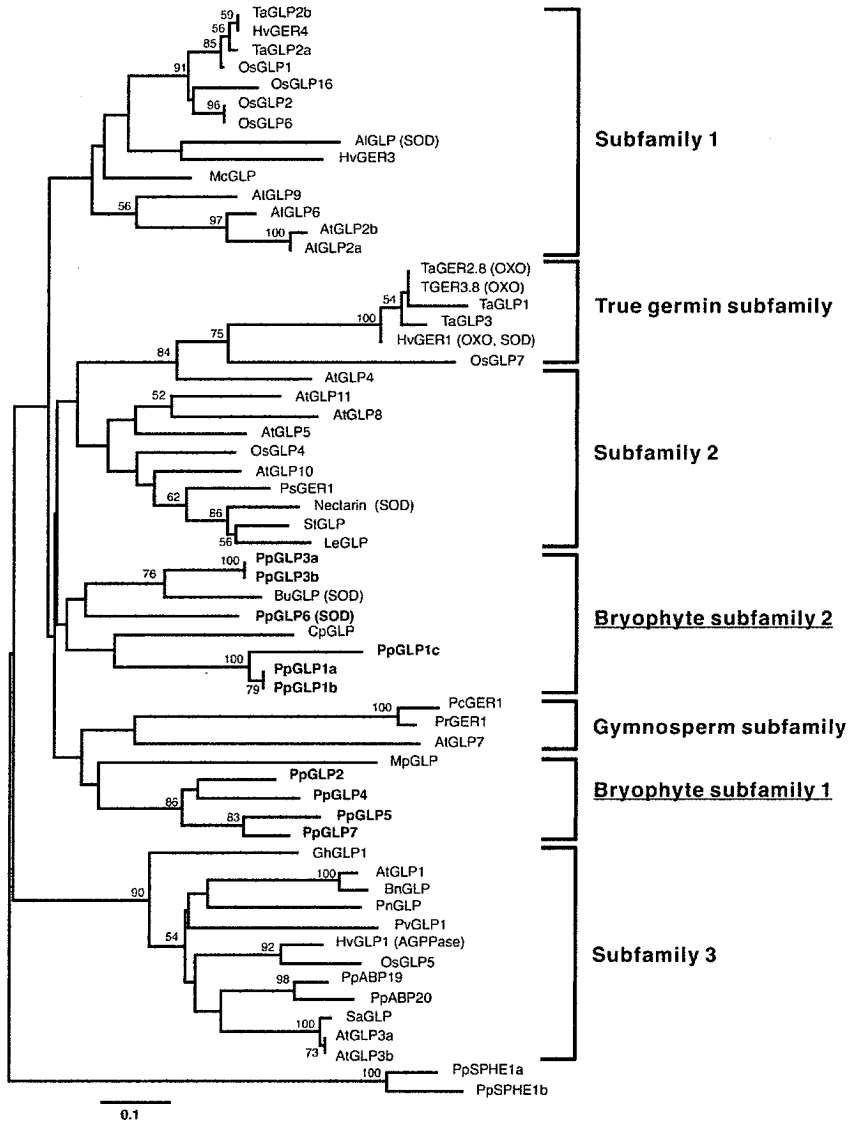
TaGLP2b
HvGER4
TaGLP2a
OsGLP1
OsGLP16
OsGLP2
OsGLP6
AlGLP (SOD)
HvGER3
McGLP
AtGLP9
AtGLP6
AtGLP2b
AtGLP2a
TaGER2.8 (OXO)
TGER3.8 (OXO)
TaGLP1
TaGLP3
HvGER1 (OXO, SOD)
OsGLP7
AtGLP4
AtGLP11
AtGLP8
AtGLP5
OsGLP4
AtGLP10
PsGER1
Nectarin (SOD)
StGLP
LeGLP
PpGLP3a
PpGLP3b
BuGLP (SOD)
PpGLP6 (SOD)
CpGLP
PpGLP1c
PpGLP1a
PpGLP1b
PcGER1
PrGER1
AtGLP7
MpGLP
PpGLP2
PpGLP4
PpGLP5
PpGLP7
GhGLP1
AtGLP1
BnGLP
PnGLP
PvGLP1
HvGLP1 (AGPPase)
OsGLP5
PpABP19
PpABP20
SaGLP
AtGLP3a
AtGLP3b
PpSPHE1a
PpSPHE1b
0.1
Subfamily 1
True germin subfamily
Subfamily 2
Bryophyte subfamily 2
Gymnosperm subfamily
Bryophyte subfamily 1
Subfamily 3

Fig. 14B

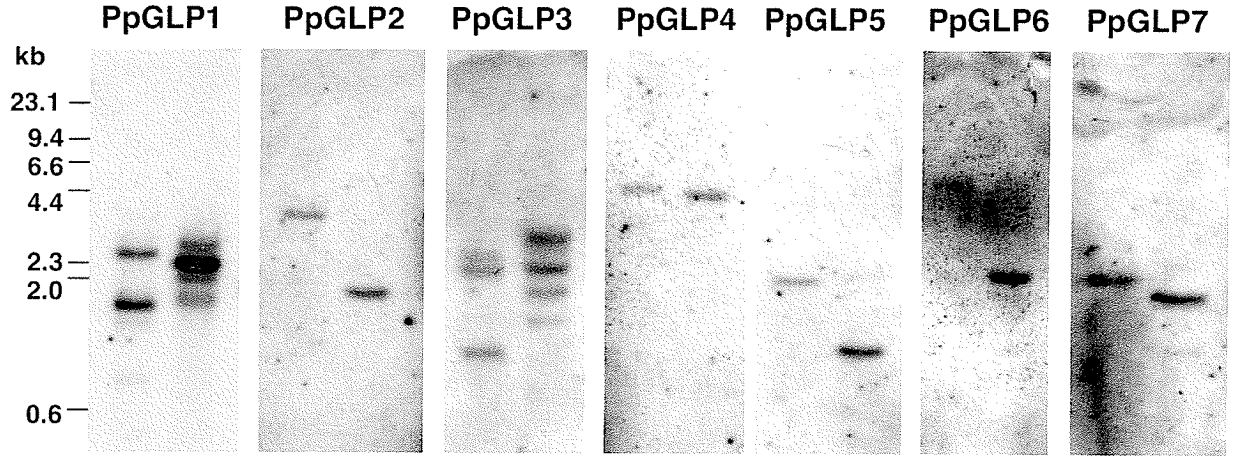

Fig. 15 Genomic Southern blot analysis of *PpGLP*. Genomic DNA (15 μg) was digested with *Eco*RI (left lane of each panel) or *Hin*dIII (right lane of each panel) and hybridized with *PpGLP*-specific probes. Lengths of molecular size standards are indicated on the left in kbp.

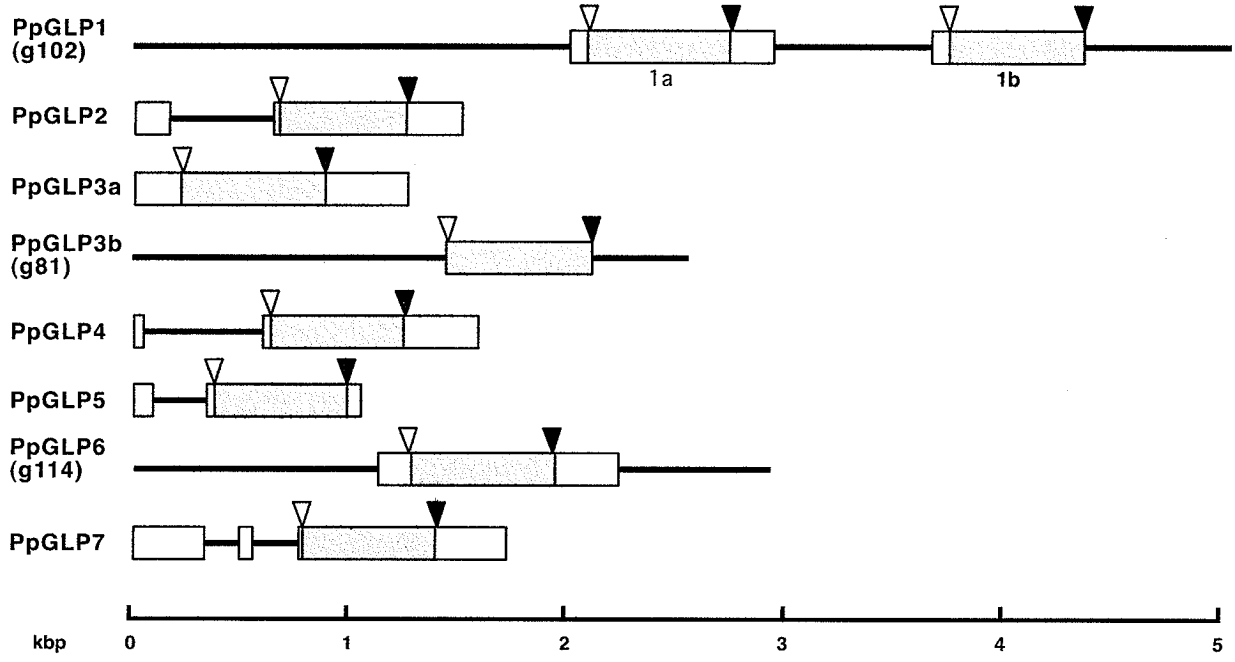

Fig. 16 Structure of *PpGLP* genes. Three genomic sequences obtained from the genomic library and 5 genomic PCR products are shown. Phage clone numbers are shown in parentheses. The regions identical to cDNA, open reading frame, initiation codon, and termination codon are indicated by opened boxes, closed boxes, opened arrows, and closed arrows, respectively. The 5'-untranslated region of *PpGLP3b* and the 3'-untranslated regions of *PpGLPs 1b* and *3b* have not been identified. The DDBJ/EMBL/GenBank accession numbers of the sequences used for this analysis were: PpGLP1 (g102), AB177646; PpGLP2, AB185322; PpGLP3a, AB177349; PpGLP3b (g81), AB177645; PpGLP4, AB185323; PpGLP5, AB185324; PpGLP6 (g114), AB185492; PpGLP7, AB185325.

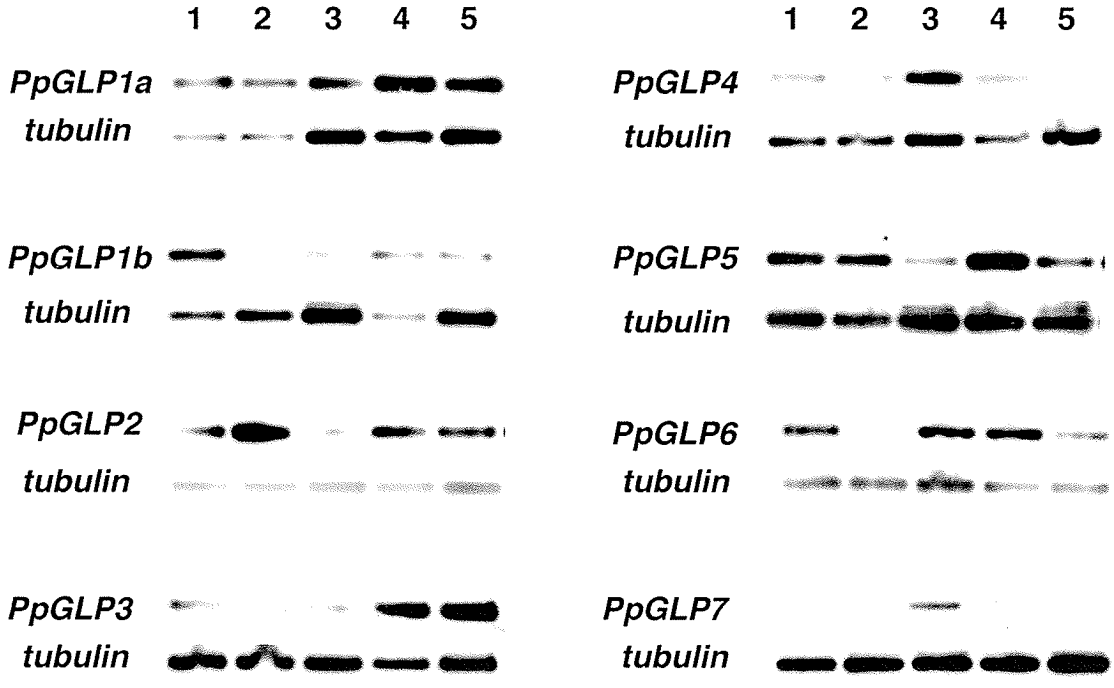

Fig. 17 The effect of plant hormones on the expression of *PpGLPs*. Total RNA extracted from *P. patens* grown for 10 days on the BCDATG plate containing 1 μM each plant hormone was used for RT-PCR. Lane 1 shows control with no hormone treatment. Lanes 2, 3, 4, and 5 represent use of RNA extracted from NAA, BA, GA_3, and ABA treated plants, respectively. Amplifications of *PpGLPs 1a*, *1b*, *3*, and *4*, and *PpGLPs 2*, *5*, *6*, and *7* were performed for 13 and 15 cycles, respectively. Alpha tubulin was amplified in the same reaction and used as an internal control.

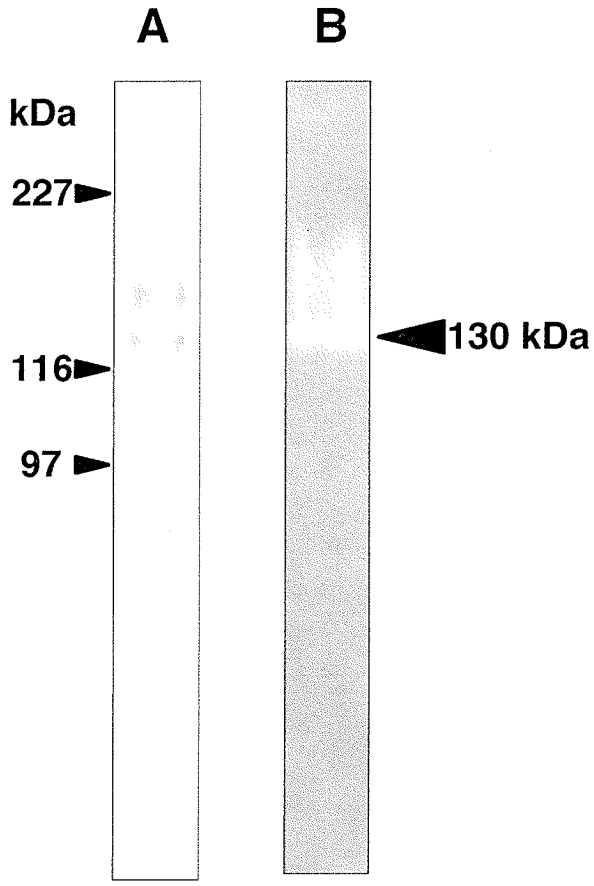

Fig. 18 Purification of extracellular Mn-SOD from *P. patens*. An aliquot (20 μL) of purified protein was separated by nondenaturing SDS-PAGE. CBB staining and SOD activity staining are shown in A and B, respectively. Molecular weights of standards are indicated on the left in kDa.

CONCLUSION

In this study, I performed expression analysis of BuGLP taking note of its SOD activity and comprehensive analysis of GLP family in *P. patens*. In chapter I, I proposed that salt stress and H_2O_2 signal are transmitted through independent pathways at least in part, and regulate *BuGLP* transcription oppositely. In chapter II, two novel groups of bryophyte GLP were identified by phylogenetic analysis of PpGLP and their plant hormone responsive expression were referred. I discuss physiological functions of GLP and their evolution.

Functions of GLP as a cell wall protein

The plant cell wall is largely composed of polysaccharides (cellulose, hemicelluloses and pectins), enzymes, structural proteins and phenolic esters (Carpita and Gibeaut, 1993). Many wall-modifying proteins have been characterized, including pectinases, expansins, and xyloglucan endotransglycosylases (Campbell and Braam, 1999). Recently, ROS produced in cell wall are essential for cell wall loosening and elongation during growth (Schopfer et al., 2002; Liszkay et al., 2004). Germins and GLPs were identified as cell wall proteins and biochemical studies to date demonstrate three distinct enzymatic activities among the family. Both OXO activity and SOD activity generate H_2O_2 that serves as a bifunctional molecule in cell wall modification. Hydroxyl radicals ($^{\cdot}OH$), which derived from H_2O_2 by Haber-Weiss reaction, cleave cell wall polysaccharide and result in cell wall loosening (Schopfer et al., 2002), while H_2O_2 is used for peroxidase catalyzed cross-linking between cell wall and cell wall

proteins and causes cell wall stiffening. Another enzymatic activity is AGPPase activity that catalyzes hydrolytic cleavage of ADP-glucose and contributes to production of substrate for cell wall polysaccharide biosynthesis (Rodríguez-López et al., 2001). These suggest that germins and GLPs with enzymatic activities play roles to modify cell wall structure.

In mammalian cells, extracellular matrix (ECM) adhesive proteins containing Arg-Gly-Asp (RGD) motif interact with plasma membrane proteins known as integrins, which are transmembrane proteins and bind to actin cytoskeleton via other anchor protein, resulting in physical coupling between cytoskeleton and ECM (Giancotti and Ruoslahti, 1999). In plants, the linkages between plasma membrane and cell wall are also essential for proper growth and development (Baluska et al., 2003). Plants lack structural homolog of integrin in their genome and plant proteins playing a role as linker molecules instead of integrin are not revealed, but some of plasma membrane associated proteins such as cell wall-associated kinases and arabinogalactan proteins are thought to be candidates for functional homolog of integrin. Although higher plants also seem to use RGD-containing proteins to connect cell wall with plasma membrane (Mellersh and Heath, 2001; Baluska et al., 2003), little is known about the linker proteins involved in the plasma membrane-cell wall adhesion. Because a number of GLPs have RGD motif and all of them are thought to be extracellular proteins (Fig. 13; Berna and Bernier, 2001), those GLPs could be candidates for linker proteins. Cell wall-associated defence responses such as H_2O_2 production require plasma membrane-cell wall adhesion (Mellersh and Heath, 2001). Papilla-specific accumulation of barley GLP during defense response may serve as a supply of linker protein in penetration point to connect plasma membrane with cell wall (Wei et al.,

1998). Lane et al (1992) found that interaction between germin and cell wall polysaccharide. These observations suggest that some members of GLPs act as adhesion protein that interacts with polysaccharide of cell wall and plasma membrane.

Hormonal signaling in bryophytes

The most characterized ABA response in higher plants is stomatal closure. Mosses also have stomata that respond to ABA (Garner and Paollilo, 1973), while these are confined to the sporophyte. The role of ABA in mosses has received little attention, however ABA-inducible wheat *Em* promoter was activated by ABA in *P. patens* through specific binding of transcription factor to ABA-responsive element (Knight et al., 1995) and ABA-induced freezing tolerance with characteristic gene expression pattern were observed in *P. patens* (Minami et al., 2003). In this study, I proposed that H_2O_2 and Ca^{2+} influx may be involved in ABA signaling pathway in *B. unguiculata* cultured cells (Fig. 11). This pathway is well-characterized in guard cells of higher plants. ABA represses seed germination and GA promotes it in higher plants. Mosses and liverworts grown on GA_3 containing medium showed enhanced formation of gametophore and antheridia, while ABA inhibits both of them (Kumra and Chopra, 1986; Chopra and Mehta, 1987). This suggests that the antagonistic interaction of ABA and GA signaling is also present in bryophytes. The role of wheat germin during germination has been thought to be involved in cell wall relaxation after water imbibition and defending seed against pathogen by generating H_2O_2 from oxalate. PpGLP6 and BuGLP are extracellular Mn-SODs that generate H_2O_2 from O_2^- and their expression is suppressed by ABA. Further, PpGLP6 was induced by GA_3 treatment (Fig. 17). This supports existence of the crosstalk between ABA and GA, and may

suggest that extracellular Mn-SOD promotes 'seed germination' via cell wall loosening by H_2O_2 generation in mosses. Although the developmental stage of mosses corresponding to seed germination of higher plant is not clear, the restriction of the protonemata formation of spore in the capsule seems to be similar to the seed dormancy of higher plants. The inhibition of protonemata formation of spore in the capsule and its release could be regulated by ABA and GA.

In spite of several effects of GA_3 in bryophytes have been reported, their action mechanisms for GA are scarcely clarified. A few genes homologous to those encoding GA biosynthetic enzymes of higher plants were observed in *P. patens* EST database that consists of transcripts expressing in gametophytes (Nishiyama et al., 2003), suggesting biosynthesis of GA in gametophyte of *P. patens*. The promotion of elongation growth by GA_3 was reported in seta of liverwort as well as in higher plants (Asprey et al., 1958), whereas obvious elongation growth by GA_3 in gametophyte of *P. patens* was not observed (data not shown). Hormonal responses of sporophytes seemed similar to that of higher plants. This indicates that hormone-responsive genes were under different regulations between sporophyte and gametophyte. Six of 16 classes of plant-specific transcription factors are not found in gametophytic EST database of *P. patens* (Nishiyama et al., 2003). Absence of those transcription factors may be responsible for defective of hormonal responses in gametophyte. I'm interested in whether those transcription factors express in sporophyte. Clarification of action mechanisms of plant hormones in bryophytes would lead to understanding of the origin and evolution of plant hormone responses in land plants.

Evolution of GLP family

GLPs have not been found from charophycean green algae that are considered as the origin of land plants (Kenrick and Crane, 1997; Qiu and Palmer, 1999). Transition of biotope from water to air exposed plants to new environmental conditions that resulted in physiological and structural changes of cell wall (Kenrick and Crane, 1997). Acquisition of primitive GLP thought to have occurred in those days. In phylogenetic analysis of GLPs, novel class of GLPs that are clearly distinguished from higher plant GLPs are found from bryophyte GLPs (Fig. 14). This is supported by analysis of their genomic structure (Fig. 16). MpGLP isolated from liverwort lacked 12 amino acids in the same region of short PpGLP and belonged to bryophyte subfamily 1, even if only cupin domain was used for the phylogenetic analysis (Fig. 14B). In addition, long PpGLPs and other moss GLPs are classified into bryophyte subfamily 2 that are more similar to higher plant GLPs than to short types, indicating phylogenetic distances between short and long PpGLPs. GLPs with Mn-SOD activity are found in mosses and higher plants. In spite of the existence of GLP in liverwort (Fig. 14B), extracellular Mn-SOD activity was not detected from extracellular protein fraction of a liverwort, *Marchantia paleacea* var. *diptera* (Nakata, unpublished result). Moreover, all GLPs with Mn-SOD activity were identified as long GLP. These data suggest that ancestor of land plants acquired protein that resembles to short PpGLPs, followed by acquisition of long GLP. Long GLP that encode extracellular Mn-SOD would emerge after divergence of mosses and liverworts. During evolution of plants, GLP family widely distributed among land plants and some of them, such as BuGLP from *B. unguiculata* and Nectarin I from tobacco, have retained SOD activity, whereas most of them have lost SOD activity. Finally, several cereal germins acquired OXO activity after divergence of monocotyledonous and dicotyledonous plant.

A characteristic feature of mosses is the alternation of two generations, gametophyte and sporophyte. The presence of a multi-cellular gametophyte in bryophyte invites research on putative differences between gametophytic and sporophytic gene regulation (Reski, 1998). Although physiological roles of short GLP is not clarified, I speculated that short GLPs would also be involved in cell wall modification of sporophytic tissues such as protonema. Here, I suggest a hypothesis that both short and long GLPs were functional in multi-cellular gametophyte in bryophytes, whereas short GLPs have lost their physiological roles during the evolution of plants. This could relate to advent of lessened gametophyte and well-developed sporophyte in higher plants in which only long GLPs are functional. In this context, it is interesting that *Arabidopsis* GLPs are all long GLPs (Carter et al., 1998) and short GLP genes have been lost from the genome. Predominant expression of short PpGLPs compared to that of long PpGLPs in gametophyte (Table 3 and Fig. 14) also supports the hypothesis.

I analyzed bryophyte GLPs throughout my study for purpose of understanding of their physiological roles. The results obtained in this study are basic data for investigation about GLPs with molecular genetic techniques. I am generating transgenic plants that overexpress PpGLP and PpGLP-knockout lines of *P. patens*. GLPs would play essential roles for various functions of cell wall because they have numerous numbers of homologous genes and different enzymatic activities. In the future, elucidation of physiological functions of GLPs by molecular genetic analysis will provide new insight into regulation of cell wall functions.

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