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Two Proton Pump Interactors Identified from a Direct Phosphorylation Screening of a Rice cDNA Library by Using a Recombinant BRI1 Receptor Kinase

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Abstract

A direct phosphorylation screening of a rice cDNA library resulted in isolation of 35 BIP clones encoding brassinosteroid receptor kinase (BRI1)-interacting proteins. Among the candidate substrates for BRI1, two clones were found to encode similar proton pump interactor proteins homologous to *Arabidopsis* PPI1, which was reported to interact with a regulatory region of plasma membrane H^+-ATPase. The rice proton pump interactors BIP103 and BIP131 contained 627 and 621 amino acids, respectively, with carboxyl-terminal hydrophobic region characteristic of tail-anchored proteins. Northern blotting analysis indicated that mRNAs for both interactors increased significantly after brassinolide treatment of lamina joint cells, which are especially sensitive to exogenous brassinosteroids.

Accession numbers: AB117987, AB117988, AB117989, AB117990, AB117991, AB117992, AB117993, AB117994, AB117995, AB117996, AB117997, AB117998, AB117999, AB118000, AB118001, AB118002, AB118003, AB118004, AB118005, AB118006, AB118007, AB118008, AB118009, AB118010, AB118011, AB118012, AB118013, AB118014, AB118015, AB118016, AB118017, AB118018, AB118019, AB118020, AB118021.

Key words: brassinosteroid, BRI1, H^+-ATPase, lamina joint, phosphorylation screening, protein interaction, proton pump interactor, receptor kinase, rice.

Abbreviations

ATPase, adenosine triphosphatase; BIP, BRI1-interacting protein; BR, brassinosteroid; BRI1, brassinosteroid insensitive 1; BRI1-KD, BRI1-kinase domain; GST, glutathione *S*-transferase; ORF, open reading frame; OsBRI1, *Oryza sativa* BRI1; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription followed by PCR; UTR, untranslated region.

Introduction

Brassinosteroid (BR) is a plant steroid hormone involved in a variety of aspects of cell growth and morphogenesis (Clouse and Sasse, 1998). Analyses of the mutants defective in the biosynthesis and perception of BRs have identified new genes responsible for metabolic and signal transduction pathways (Bishop and Yokota, 2001; Jonak and Hirt, 2002). Identification of *BRI1* gene responsible for BR-insensitive dwarf mutant of *Arabidopsis thaliana* (Li and Chory, 1997) resulted in the progression of studies on these hormone responses (Li, 2003). *BRI1* gene product was characterized as a typical plant receptor kinase with extracellular leucine-rich repeat (LRR) and cytoplasmic kinase domains. The LRR domain binds to brassinolide, the most active BR, and the kinase domain is phosphorylated *in vivo* (Wang *et al.*, 2001). Rice BRI1 homologue (*OsBRI1*) was cloned and shown to complement a dwarf mutant d61 (Yamamuro, *et al.*, 2000). From these studies, BRI1 has been established to be a unique plant receptor kinase. Another BR-insensitive locus *BIN2* (Li *et al.*, 2001)

of *A. thaliana* coding for GSK3-like kinase is suggested to act as a negative regulator by phosphorylating and destabilizing the positive regulators BZR1 and BES1, which were identified from *bri1* suppressor mutants (Wang *et al.*, 2002; Yin *et al.*, 2002). BRs are considered to induce the accumulation of BZR1 and BES1 by inhibiting the negative regulator BIN2 (He *et al.*, 2002).

BRI1 associated receptor kinase 1 (BAK1) was isolated successfully from *Arabidopsis* cDNA library by yeast two-hybrid screening (Nam and Li, 2002; Li, 2003). Overexpression of *BAK1* gene rescued a weak *bri1* mutant. BRI1 and BAK1 were shown to activate each other through transphosphorylation in yeast cells and associate physically in plant cells. The heterodimeric kinases may be responsible for BR perception, but other substrate proteins downstream of BRI1 have not been reported. We have employed an *in vitro* phosphorylation screening method to isolate the cDNAs coding for the downstream factors expecting to connect the kinases to the target proteins directly involved in the BR effects such as cell elongation.

Materials and Methods

Plant material

Rice (*Oryza sativa* cv. Nipponbare) seeds were soaked in 0.5% benomyl hydrate and imbibed for 24 h at 30℃. The seeds were washed with distilled water several times and then germinated in a light or dark chamber at 30℃ for 7 days. The lamina joints (5 mm in length) at the second leaf sheath of individual plant were excised, kept in the light or dark for 1 more day in distilled water and used for total RNA extraction. For Northern blot analysis, lamina joints were treated with further 1 μM brassinolide in the dark (Wada *et al.*, 1981).

Plasmid construction

A part of *Arabidopsis* BRI1 cDNA (nt 2455 to 3591) containing the kinase domain (Lys-819 to Leu-1196, Li and Chory, 1997) was amplified with a primer set BRI-F02 (5′-TTAAGGATCCAAGAG ACGGAGAAAGAAAGA-3′; *Bam*HI site is underlined) and BRI-R02 (5′-GCGGAATTCTTAT CATAATTTTCCTTCAGG-3′; *Eco*RI site is underlined) from *Arabidopsis* bud cDNA. The PCR product was cloned into an expression vector pGEX-6P-1 (Amersham Biosciences) to construct pGEX-6P-1-BRI1-KD for the production of GST-BRI1-KD. For the expression of a mutant kinase, PCR-based site directed mutagenesis was carried out by using pGEX-6P-1-BRI1-KD as a template and four primers: BRI-F03 (5′-TAAACTGGTCCA CACGGCGGAAG-3′), BRI-MF01 (5′-TACGG TGTGGTCTTACTCAAGCTACTCACG-3′, mutation site is underlined), BRI-MR01 (5′-CGTTT ACCCGTGAGTAGCTTGAGTAAGACC-3′, mutation site is underlined) and BRI-PGEX-R (5′-GTCCTGCAACTTTATCCGCCTCC-3′, vector sequence). The internal *Pst*I fragment (1574 bp) of the PCR product was introduced into the corresponding region of pGEX-6P-1-BRI1-KD to construct pGEX-6P-1-BRI1-KDM for the production of GST-BRI1-KDM, which had 1078-Glu to 1078-Lys change originally found in the mutant allele *bri1-101* (Friedrichsen *et al.*, 2000).

The rice BRI1 cDNA encoding the kinase domain (Yamamuro *et al.*, 2000) was also obtained by RT-PCR with OsBRI-F (5′-CGATCTCGAGATCATC ATAGCCATTGGGAG-3′; *Xho*I site is underlined) and OsBRI-R (5′-GCGCGGCCGCGTTGTTTCT AATCCTTCTCC-3′; *Not*I site is underlined) primers and cloned into pGEX-6P-1 to construct pGEX-6P-1-OsBRI1-KD.

Production of recombinant protein

The recombinant protein was produced in *Escherichia coli* strain XL10-GOLD (Stratagene) and purified by using Glutathione Sepharose beads (Amercham Biosciences) as described previously (Matsushita *et al.*, 2000). The protein beads were suspended in NETN-D buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% Nodidet P-40, 1 mM DTT) and stored at 4℃.

To remove the GST moiety of GST-BRI1-KD and GST-BRI1-KDM, the fusion proteins were immobilized on Glutathione Sepharose beads and incubated for 15 h at 4℃ in 50 μl NETN-D containing 2 U PreScission Protease (Amersham Biosciences) to prepare BRI1-KD and BRI1-KDM.

Construction of a cDNA library

The total RNA was extracted from rice lamina joints with ISOGEN (Nippon Gene, Tokyo). The mRNA was purified with PolyA Tract mRNA Isolation System (Promega) and used for cDNA synthesis with TimeSaver cDNA synthesis Kit (Amersham Biosciences). The cDNA was subjected to ligation to an expression lambda phage vector λGEX5 (Fukunaga and Hunter, 1997; Matsushita *et al.*, 2001) by using phosphorylated oligonucleotide adapters (5′-AGGTGCTGG-3′ and 5′-CCAGCA CCTGCA-3′) and an *in vitro* packaging kit (Epicentre Technologies) to yield a cDNA library consisting of 1.4×10^7 independent clones.

Phosphorylation screening

The recombinant protein BRI1-KD was used for

a solid-phase phosphorylation screening (Fukunaga and Hunter, 1997) of the rice cDNA expression library. After infection of *E. coli* BB4, phage plaques were overlaid with nitrocellulose membrane (BA-S 85, Schleicher & Schuell) that had been soaked in 10 mM isopropyl-β-thiogalactopyranoside and incubated for 4 h at 37℃. The membranes were treated with a rinse buffer (10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 0.1% Tween 20, 1 mM DTT) and incubated for 16 h at 4℃ in a blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 3% skim milk, 1 mM EDTA). After extensive washing with a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, the membranes were rinsed with a BRI1-KD reaction buffer (BRB) consisting of 20 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 μM Na_3VO_4, 5 mM β-glycerophosphate, 5 mM NaF, 0.1% Triton X-100 and 2 mM DTT. To avoid non-specific binding of ATP or autophosphorylation, which should generate false positive clones, the membranes were incubated for 60 min at 30℃ in BRB containing 25 μM cold ATP. After washing with BRB without ATP, the membranes were incubated for 60 min at 30℃ in BRB containing [γ-^{32}P] ATP (7.4 GBq mmol^{-1}, 185 kBq ml^{-1}) and the recombinant kinase BRI1-KD (1 μg ml^{-1}). The membranes were washed 7 times each for 5 min with a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 20 mM NaF, 0.1% Triton X-100, and twice each for 10 min with the same buffer without Triton X-100. Positive clones were identified by autoradiography on BAS-1500 (Fuji film). The screening was repeated for purification of positive clones (λ BIP101 to λ BIP135).

For further analyses phage DNA was digested with *Not*I followed by self-ligation to recover corresponding expression plasmid (pGEX-PUC-3T-λBIP101 to pGEX-PUC-3T-λBIP135) for the production of GST-fused protein (GST-λBIP101 to GST-λBIP135). The nucleotide sequences of cDNA inserts were determined by using the vector primers pGEX2(5′-CTGGTTCCGCGTGGATCCCC-3′) and pGEX5 (5′-ATTTCCCCGAAAAGTGCCAC-3′).

In vitro phosphorylation assay

The recombinant GST-fused proteins derived from positive clones were purified by an affinity binding on Glutathione Sepharose. For phosphorylation assay, 500 ng of the purified protein immobilized on the Glutathione Sepharose beads was incubated with 20 ng of BRI1-KD or BRI1-KDM for 30 min at 30℃ in BRB containing [γ-^{32}P] ATP (5.6 TBq mmol^{-1}, 3.7 MBq ml^{-1}). After washing four times with NETN-D buffer, the GST-fused protein was eluted for 10 min at 65℃ in a sample buffer for SDS-PAGE. The extent of phosphorylation was measured on BAS-1500.

In vitro protein binding assay

The GST-fused proteins (500 ng each) were eluted from Glutathione Sepharose beads with Tris-HCl (pH 8.5) containing 10 mM glutathione and blotted onto nitrocellulose membrane by using a slot blotter. After blocking for 30 min at 4℃ with a blocking buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM $MgCl_2$, 0.1% Triton X-100, 3% skim milk, 0.1% NaN_3 and 1 mM DTT), the membranes were incubated for 4 h at 4℃ in the same buffer containing the probe protein BRI1-KD (900 Bq ml^{-1}) that was labeled with [γ-^{32}P] ATP by autophosphorylation. The membrane was washed four times with a washing buffer (10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, 0.2% Triton X-100, pH 7.4) and subjected to autoradiogram on BAS-1500.

PCR and sequence analyses of proton pump interactor cDNAs

To determine the full-length cDNA sequences for proton pump interactor BIP103 and BIP131, cDNA fragments covering upstream regions were amplified by PCR using the rice lamina joint cDNA library as a template. For BIP103 and BIP131, 1979 bp fragments (1 to 1979) containing each complete ORF were amplified by using the upstream primers BIP103-F00 (5′-ATGGGAATGGAGGTTGTTGGAACTGAGGCTGCACCAG-3′) or BIP131-F00 (5′-ATGGAGGCCACTGGTGCTGAAGCTACATTGT-3′) in combination with the downstream primers BIP103-R00 (5′-CAGTCCACGTCTCAGCAGACCACGCACTCTG-3′) or BIP131-R00 (5′-GAACAGGAACCATGTAGGGGATCCTCATTCAG-3′), respectively. Upstream overlapping fragments of 767 bp (-95 to 672 of BIP103) or 781 bp (-118 to 663 of BIP131) containing each 5′ UTR were amplified by nested PCR using the first primer set pGEX2 (5′-CTGGTTCCGCGTGGATCCCC-3′, vector sequence) and BIP103-R00 or BIP131-R00, and the second set pGEX3 (5′-GGGAATTTCCGGTGGTGGTG-3′, vector sequence) and BIP103-R08 (5′-AAAGACCAGACCGAAAGTCATCTCC-3′) or BIP131-R08 (5′-TGGAACCAGGTCAAAGGTTATTGAG-3′).

These PCR products were cloned into pGEM-T Easy vector (Promega) to construct pGEM-T Easy-BIP103-1 and pGEM-T Easy-BIP131-1 con-

taining complete ORF, and pGEM-T Easy-BIP103-2 or pGEM-T Easy-BIP131-2 containing 5′ UTR. Nucleotide sequences determined from these plasmids were used to reconstruct the full-length sequences of BIP103 and BIP131 cDNAs.

Northern blotting analysis

Total RNA was extracted from rice lamina joints with TRIzol reagent (Invitrogen). The RNA was separated on 1.2% agarose gel containing 0.66 M formaldehyde and blotted onto Immobilon Ny^+ membrane (Millipore). The membrane was prehybridized for 3 h at 42℃ in the prehybridization buffer containing 50% formamide, 5x SSC, 50 mM sodium phosphate buffer (pH 6.8), 0.2% SDS, 5x Denhardt's solution and denatured salmon sperm DNA (0.1 mg ml^{-1}). Hybridization was carried out for 16 h at 42℃ in the hybridization buffer containing 50% formamide, 5x SSC, 50 mM sodium phosphate buffer (pH 6.8), 0.2% SDS, 0.5x Denhardt's solution and denatured salmon sperm DNA (0.1 mg ml^{-1}), 10% dextran sulfate and [α-^{33}P] dCTP-labeled DNA probe (53 kBq ml^{-1}). The membrane was washed six times at room temperature with 2x SSC containing 0.1% SDS and twice for 15 min at 50℃ with 0.2x SSC containing 0.1% SDS.

Results

Activity of recombinant BRI1 kinase

The recombinant kinase domain (378 amino acids) of the receptor BRI1 revealed significant autophosphorylation activity irrespective of the presence of amino-terminal GST fusion. The activity was enhanced 5-10 times by the addition of 10 mM $MnCl_2$ in the reaction mixture (data not shown). On the other hand, a mutant kinase GST-BRI1-KDM showed complete loss of the autophosphorylation activity (data not shown).

Phosphorylation screening for BRI1 substrate proteins

Phage expression cDNA library of rice lamina joint was screened for the substrate proteins for BRI1 kinase (BRI1-KD) on nitrocellulose membrane with immobilized phage plaques. Among $3.1x10^5$ total plaques, 53 clones were shown to encode GST-fused proteins that were phosphorylated *in vitro* by BRI1-KD. Sequencing analysis of the positive clones identified overlapping cDNAs and allowed to reconstruct 35 cDNAs of distinct genes, whose products were designated as BRI1-interacting proteins (BIP) as shown in **Table 1**.

Phosphorylation and binding assays

Plasmid clones derived from positive phage clones were used to express GST-fused proteins encoded by cDNA inserts. These recombinant proteins were purified from *E. coli* lysate and used for *in vitro* phosphorylation and binding assays with BRI1-KD. As shown in **Fig. 1**, all proteins (GST-λBIP101 to GST-λBIP135) except GST-λBIP114 showed BRI1-KD dependent phosphorylation. The phosphorylation of BIP114, which was identified as a nucleotide diphosphate kinase, occurred in the presence of BRI1-KD as well as BRI1-KDM indicating the autophosphorylation activity of the cDNA product itself.

During the course of our project, rice BRI1 cDNA was reported by Yamamuro *et al.* (2000). Using the nucleotide sequence information, we isolated rice BRI1 cDNA and prepared a recombinant kinase (OsBRI1-KD) to confirm the results obtained with *Arabidopsis* BRI1. We selected 18 clones as representatives showing from low (+) to intense (++++) phosphorylation described in **Table 1**. Using OsBRI1-KD, we performed phosphorylation assays with the recombinant proteins derived from the selected 18 clones. The extents of phosphorylation of these proteins by OsBRI1-KD were comparable to those by *Arabidopsis* BRI1 shown in **Table 1** (data not shown).

The possibility for protein-protein binding between the BRI1 kinase domain and its substrate protein was examined for each phosphorylation positive clone. Among the 35 cDNAs, 13 were shown to encode proteins that also bind to the recombinant protein BRI1-KD (**Table 1**).

Data base search for cDNA identity

To identify the partial cDNA clones, whole rice genome automated annotation database (TIGR) was searched for full-length ORF. The predicted ORFs were then searched for homology in DNA databases with BLAST program (**Table 1**). The peptide sequences were also analyzed for possible transmembrane structures by using SOSUI program (Hirokawa *et al.*, 1998), which predicted 6 membrane proteins including proton pump interactor proteins BIP103 and BIP131 (**Table 1**). Membrane localization and direct interaction with BRI1 made these two similar clones more promising candidates than others. One of the BR effects is cell elongation and expansion, the mechanism of which is not known precisely yet. In the case of auxin action, the activation of plasma membrane H^+-ATPase was reported to be crucial for cell elongation (Frias *et al.*, 1996; Rober-Kleber *et al.*, 2003). Recently, the *Arabidopsis* proton pump interactor homolog

Table 1 Candidate proteins for BRI1 substrates identified by phosphorylation screening

6BRI1 interacting protein of rice[1]		Phoshoryl-ation by BRI1-KD[2]	Binding to BRI1-KD[3]	Trans mem-brane helices[4]	Homologous protein		
Name	Accession No.				Plant	Name	Accession No.
BIP103	AB117987	++++	++	1	*A. thaliana*	proton pump interactor	AJ002020-1
BIP131	AB117988	++	-	1	*A. thaliana*	proton pump interactor	AJ002020-1
BIP115	AB118002	+++	+++	2	*A. thaliana*	ATP-dependent RNA helicase like protein	AB010692-26
BIP124	AB118011	++	+	3	*A. thaliana*	ABA-and stress-inducible protein (AtHVA22a)	AF141659-1
BIP125	AB118012	++++	-	1	*Z. mays*	Em binding protein-1a	Y15165-1
BIP129	AB118016	+	-	2	*A. thaliana*	unknown protein	D84636
BIP110	AB117997	++	-	-	*A. thaliana*	ubiquitin-specific protease 23	AF302671-1
BIP112	AB117999	++	-	-	*A. thaliana*	N-hydroxycinnamoyl/ benzoyltransferase like protein	AB010072-10
BIP114	AB118001	+++[5]	-	-	*N. tabacum*	nucleoside diphoshate kinase	AB088360-1
BIP122	AB118009	+++	-	-	*A. thaliana*	FKBP-type peptidyl prolyl cis-trans isomerase	AC069474-35
BIP106	AB117993	++	-	-	*A. thaliana*	microtubule-associated protein	AL049607-8
BIP135	AB118021	+	++	-	*A. thaliana*	microtubule-associated protein	AL049607-8
BIP101	AB117989	++	+++	-	*N. tabacum*	MAR-binding protein	AB059832-1
BIP102	AB117990	+	++	-	*N. tabacum*	MAR-binding protein	AB059832-1
BIP104	AB117991	+++	-	-	*Z. mays*	SSRP1 protein	AJ244017-1
BIP105	AB117992	+++	-	-	*A. thaliana*	TATA box binding protein associated factor	T47587
BIP127	AB118014	++	-	-	*A. thaliana*	DNA-binding protein (PCMYB1)	AY087994-1
BIP133	AB118019	++	-	-	*A. thaliana*	putative transcription factor	AY033827-1
BIP113	AB118000	+++	-	-	*A. thaliana*	putative RNA-bindng protein	AL078637-10
BIP108	AB117995	++	+	-	*A. thaliana*	60S ribosomal L22-2 protein	AY088594-1
BIP123	AB118010	++	+	-	*H. vulgare*	60S ribosomal L24 protein	X94296-1
BIP107	AB117994	+++	-	-	*Z. mays*	eukaryotic translation initiation factor 5 (eIF5)	AJ132240-1
BIP126	AB118013	++	-	-	*A. thaliana*	putative formamidopyrimidine DNA glycosylase 1	AF099970-1
BIP109	AB117996	++	-	-	*A. thaliana*	unknown protein	AY088685-1
BIP111	AB117998	++	-	-	*A. thaliana*	unknown protein	AC016529-9
BIP116	AB118003	+++	+	-	*A. thaliana*	unknown protein	AY055093-1
BIP117	AB118004	++++	++	-	*A. thaliana*	unknown protein	AB024032-7
BIP118	AB118005	++++	+	-	*A. thaliana*	unknown protein	AY035182-1
BIP119	AB118006	+++	-	-	*A. thaliana*	unknown protein	AB007647-12
BIP120	AB118007	++++	+++	-	*A. thaliana*	unknown protein	AC016662-17
BIP121	AB118008	++	-	-	*A. thaliana*	unknown protein	AK118365-1
BIP128	AB118015	++	-	-	*A. thaliana*	unknown protein	AL161581-31
BIP130	AB118017	++++	++++	-	*A. thaliana*	unknown protein	AC005896-27
BIP132	AB118018	+++	-	-	*A. thaliana*	unknown protein	AY084589-1
BIP134	AB118020	++++	-	-	*A. thaliana*	unknown protein	AC022522-11

[1] Probable full-length cDNA sequences were reconstructed by RACE method for BIP103 and BIP131. Other cDNAs were determined only for the original phage clones, which were mostly partial ones.

[2] The extent of phosphorylation was measured for GST-fused proteins derived from the original partial phage clones. The autoradiograms shown in **Fig.1** were measured for photo stimulated luminescence (PSL) values and indicated as follows: + for less than 1000; ++ for 1000 to 5000; +++ for 5000 to 25000; ++++ for over 25000.

[3] Binding activities to the labeled BRI1-KD probe were measured for GST-fused proteins derived from the original partial phage clones. The PSL values are indicated as follows: − for 0; + for 1 to 50; ++ for 50 to 100; +++ for 100 to 300; ++++ for over 300.

[4] Probable full-length protein structures were searched in Whole Rice Genome Annotation Database (TIGR) and the numbers of transmembrane helices were predicted by SOSUI program.

[5] Phosphorylation was due to autophosphorylation activity not by BRI1-KD.

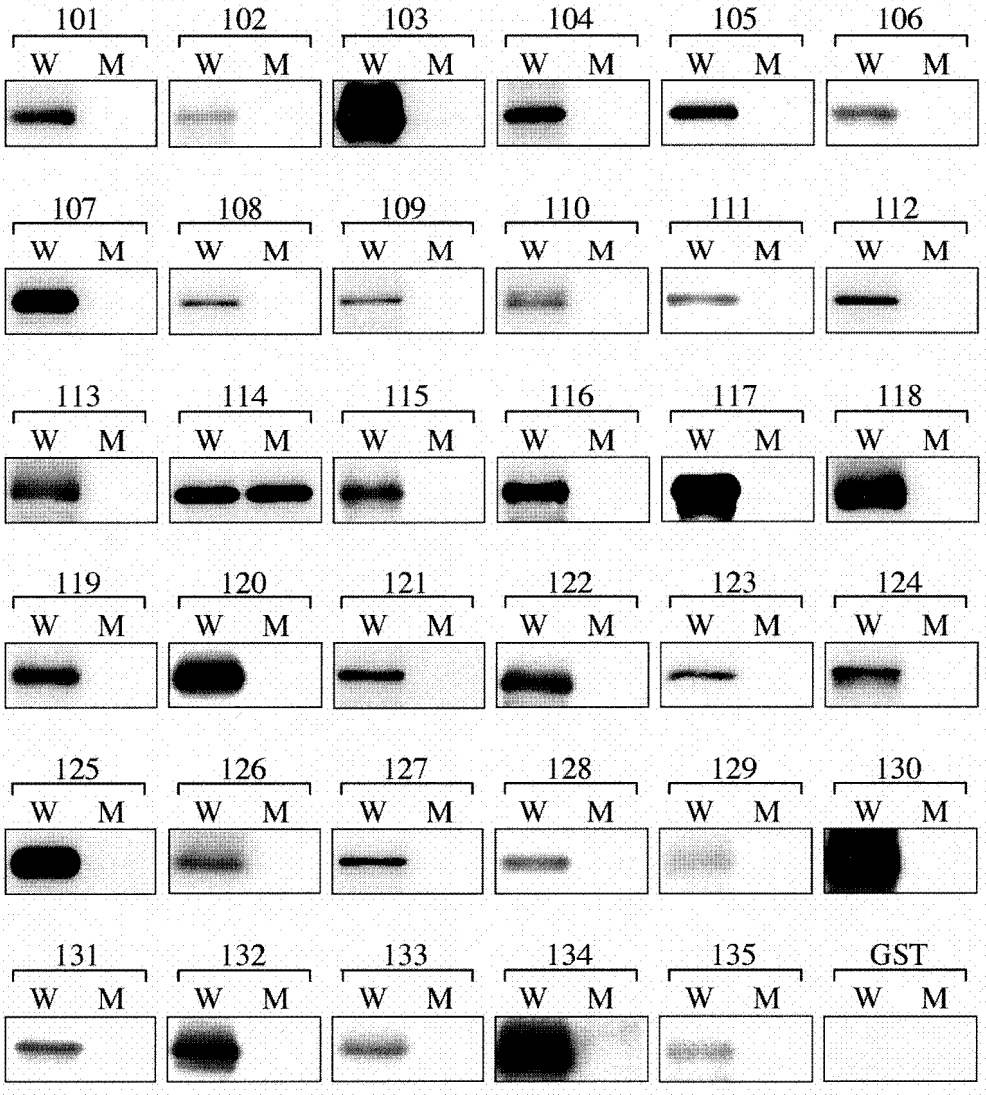

Fig. 1 Phosphorylation assays of GST-fused proteins derived from positive clones. GST-fused proteins were expressed by pGEX-PUC-3T plasmids containing the same cDNAs as those in the original phage clones. Purified proteins (GST-λBIP101 to GST-λBIP135) were incubated with wild-type (W) or mutant (M) recombinant kinase and subjected to SDS-PAGE followed by autoradiography. For each clone, samples incubated with wild-type and mutant kinases were analyzed on the same gel. The autoradiograms of protein bands are shown with clone numbers on top of lanes.

PPI1 (Morandini *et al.*, 2002) was reported to interact with the C-terminal region of the plasma membrane H^+-ATPase. For these reasons, the two clones BIP103 and BIP131 were examined preferentially further in detail.

Analysis of the full-length cDNAs for proton pump interactors

Phage clones λBIP103 and λBIP131 originally isolated by phosphorylation screening contained partial cDNA sequences with C-terminal coding sequences in addition to 3′ UTRs and poly A tails. The full-length cDNA structures reconstructed from the cDNA inserts of phage clones and PCR products established 627 and 621 amino acid sequences for BIP103 and BIP131, respectively (**Fig. 2A** and **2B**). The two amino acid sequences were 44 and 47% identical to *Arabidopsis* proton pump interactor (Morandini *et al.*, 2002) and 65% identical to each other (**Fig. 3**).

Analysis of the protein structures with SOSUI program lead to a prediction that C-terminal regions of BIP103 (Pro-599 to Tyr-617) and BIP131 (Pro-592 to Tyr-614) were highly hydrophobic and conform to a transmembrane helices.

Proton pump interactor gene expression

To examine the effect of BR on the expression of *BIP103* and *BIP131* genes, lamina joint cells were treated with brassinolide and the RNA was extracted for Northern blot analysis. As shown in **Fig. 4**, BIP103 and BIP131 mRNA levels in the tissues of dark-grown rice lamina joint increased about 2.3 times in 6 h after addition of 1 μM brassinolide. The mRNA level of OsBRI1 showed rapid and significant decrease in 2 h after brassi-

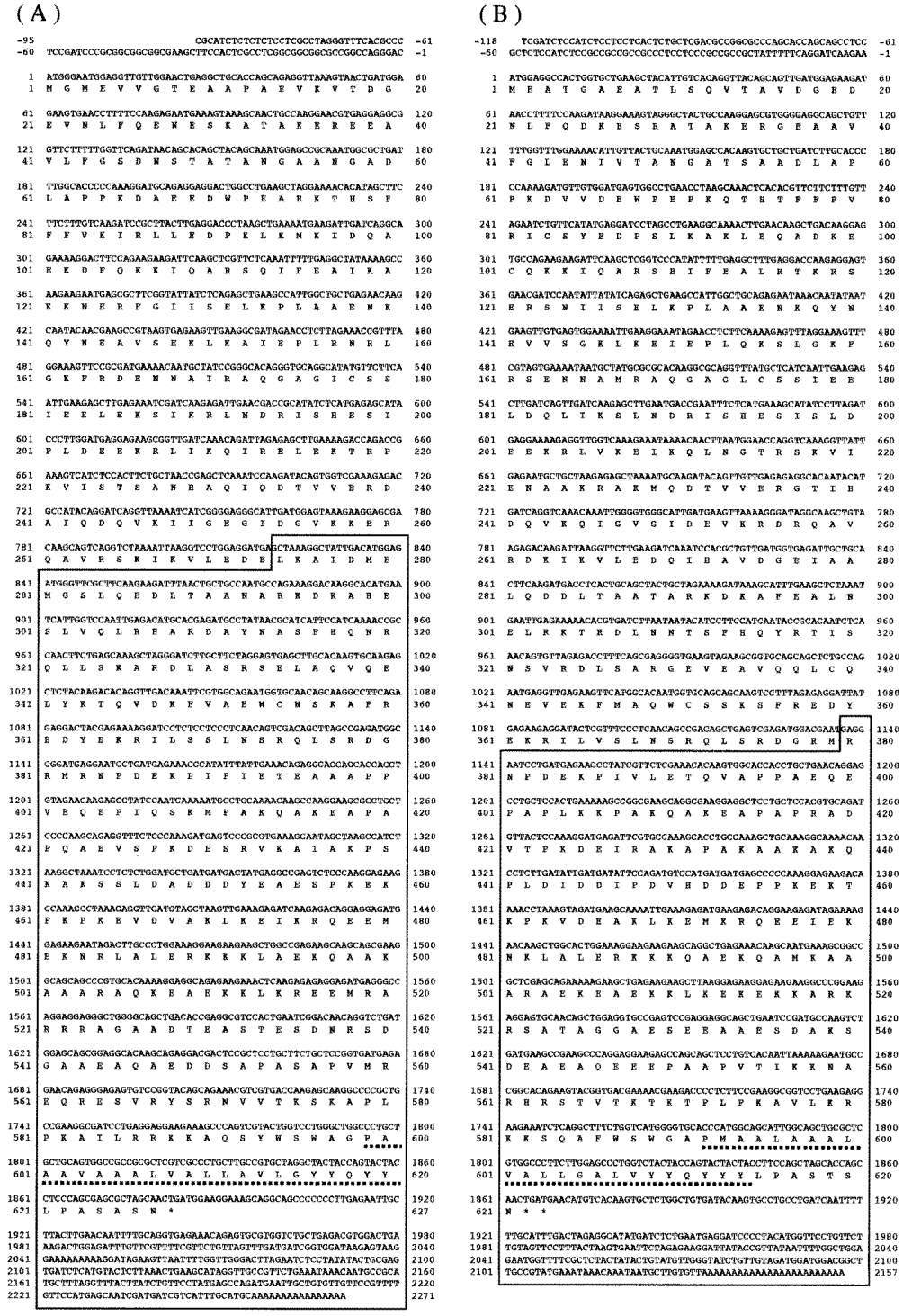

Fig. 2 DNA and deduced amino acid sequences of BIP103 and BIP131. The cDNA structures of two proton pump interactors BIP103 (A) and BIP131 (B) were reconstructed from the cDNA sequences in the original phage clones (boxed) and upstream PCR clones. The broken underlines indicated the regions predicted as transmembrane helix. Accession numbers are AB117987 (BIP103) and AB117988 (BIP131).

nolide treatment as described by Yamamuro *et al.* (2000), while the mRNA levels of a house-keeping actin gene were constant within 24 h.

Discussion

Phosphorylation screening of 3.1×10^5 phage clones resulted in identification of 35 different cDNA products, including 13 proteins showing both phosphorylation and binding with BRI1-KD. Protein factor such as BAK1 (Nam and Li, 2002; Li, 2003) was first identified by yeast two-hybrid screening for its binding ability to BRI1 and later shown to be phosphorylated by BRI1. Although such cDNA clones reported previously by other groups were not found in our positive clones, our

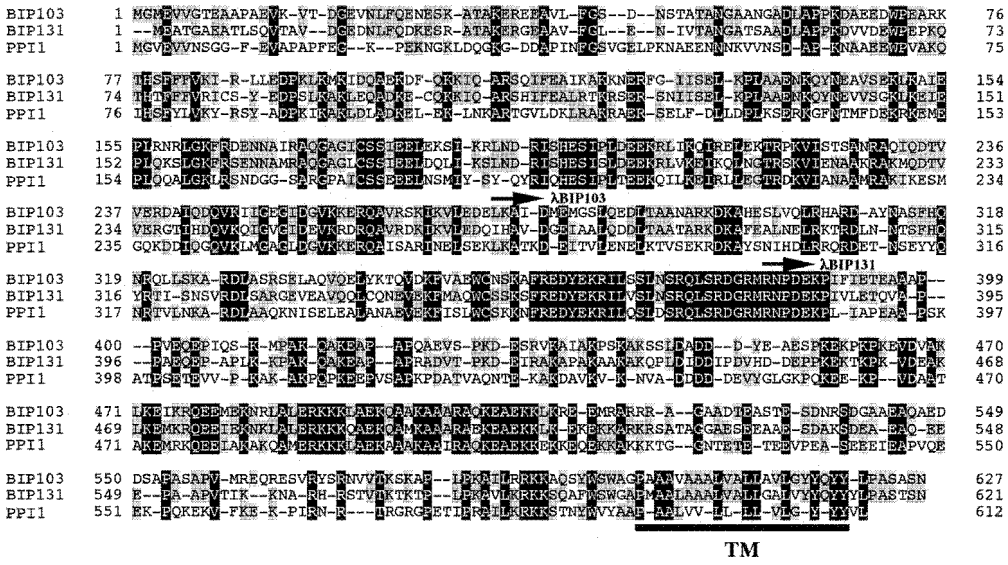

Fig. 3 Amino acid sequence alignment for the rice and *Arabidopsis* proton pump interactor proteins. Deduced amino acid sequences of BIP103, BIP131 and PPI1 (accession no. AJ002020-1) were analyzed by a program GENETYX-MAC (Software Development Co.). Amino acids conserved in all cDNAs are shown in black and those conserved in two of the three cDNAs are shown in gray. Gaps (hyphens) were introduced to maximize the homology. The start sites of peptide sequences encoded by the original phage clones were indicated by arrows. The predicted transmembrane (TM) helix were underlined.

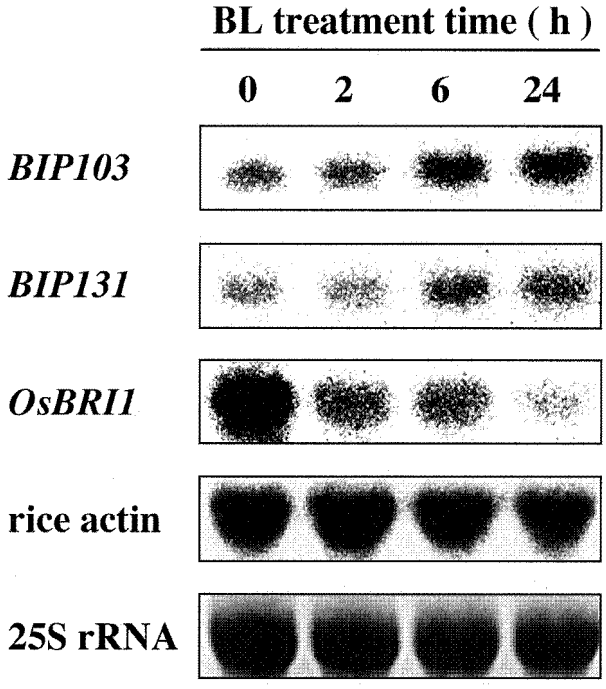

Fig. 4 Gene expression of proton pump interactors in rice lamina joint. Lamina joints were excised from rice seedlings grown in dark for 7 days at 30℃. The tissues were kept in the dark for 1 more day in water and treated with 1 μM brassinolide for additional 0, 2, 6 and 24 h. Total RNA (20 μg) was extracted from these tissues and subjected to Northern blot analysis with [α -^{33}P] dCTP labeled probe (53 kBq ml^{-1}) derived from 3′ UTR: BIP103, 2024-2245; BIP131, 1890 - 2113; OsBRI1, 2581-2976; rice actin (EST clone: S14002), 24-394.

screening method was efficient to obtain directly the candidate proteins for BRI1 substrates. Some substrates may be bound to and phosphorylated by the kinase as in the case of BAK1 and others may be released from the kinase upon phosphorylation.

Identification of cDNAs by homology search was successful to find possible homologues in other plants for all clones (**Table 1**). There were several clones of intriguing possibility to be involved in the phosphorylation cascade located downstream of BRI1. BIP107 has 79% identities with eukaryotic translation initiation factor 5 (eIF5). In mammals, eIF3 or TRIP1 is known to be physically associated with eIF5 and eIF1 and phosphorylated by type II TGF-β in mammal. Jiang and Clouse (2001) reported that the *Arabidopsis* transformant carrying antisense TRIP1 homologue (*AtTRIP1*) showed a phenotype similar to BR insensitive mutants. Thus, BIP107 could be a cofactor for TRIP1 to play some roles in growth regulation by BR. Another identifiable clone BIP124 has conserved hydrophobic stretches and 59% identities with an *Arabidopsis* abscisic acid- and stress-inducible protein AtHVA22 (Chen *et al.*, 2002), which was suggested to interact with *Arabidopsis* RHD3 (Brands and Ho, 2002) responsible for a mutation causing short and wavy root hair. This mutant phenotype was resulted from an inhibition of vacuole enlargement possibly due to a reduction of water uptake (Galway *et al.*, 1997). Although there is no evidence for cross-talk mechanisms among plant hormones involved in cell elongation and expansion, BR might exert similar

effects through BRI1 signaling.

From the phosphorylation screening, two cDNA clones similar to *Arabidopsis* proton pump interactor PPI1 were reproducibly isolated, indicating that these two clones should share conserved phosphorylation sequences. However, the GST-fused proteins derived from these original partial phage clones λBIP103 and λBIP131 differed in the extent of phosphorylation and BRI1-binding ability (**Table1**). The result of phosphorylation by OsBRI1-KD was comparable to that with BRI1-KD. The amino-terminal distal region of λBIP103, which was absent in λBIP131 and contained a highly conserved sequence to PPI1 (Phe-359 to Met-382 in **Fig. 3**) may be important for intense phosphorylation and also interaction with BRI1.

The full-length amino acid sequences of the two proton pump interactors BIP103 and BIP131 were highly homologous to each other and also to *Arabidopsis* PPI1. These proteins were predicted to have a single transmembrane region at each carboxyl terminus, which is a characteristic of tail-anchored proteins with cytoplasmic activities close to the surface of an intracellular membrane (Wattenberg and Lithgow, 2001). The amino-terminal region of PPI1 was shown to interact with the carboxyl-terminal regulatory domain of plasma membrane H^+-ATPase (PM-H^+-ATPase) leading to a stimulation of H^+-ATPase activity (Morandini *et al.*, 2002). PM-H^+-ATPase has been reported to be involved in the regulation of cell expansion or elongation by plant hormones. Goh *et al.* (1996) reported that blue-light dependent PM-H^+-ATPase activity was inhibited by abscisic acid in *Vicia* guard cells causing membrane depolarization leading to stomatal closure. Rober-Kleber *et al.* (2003) reported that auxin activates PM-H^+-ATPase resulting in apoplastic acidification contributing to cell wall loosening and cell elongation in wheat embryo. In the case of vacuolar H^+-ATPase (V-ATPase), Schumacher *et al.* (1999) reported that the *Arabidopsis det3* gene responsible for a mutant with a reduced response to BRs encodes subunit C of V-ATPase. They also described that subunit H of V-ATPase was phosphorylated *in vitro* by BRI1. Phosphorylation of V-ATPase could have some roles in the control of cell elongation by BRs. Consequently, H^+ transport through cellular membrane, irrespective of the identity of H^+-ATPase, seems to be important for the physiological action of BR.

Isolation of rice proton pump interactors among BIP clones may be implicated as possible roles in lamina inclination and internode cell elongation. A number of rice dwarf mutants have been characterized for responses to plant hormones including BR. One of such examples was found in the expression of xyloglucan endo-transglycosylase, which induces cell wall loosening leading to cell elongation (Uozu *et al.*, 2000). Our Northern blot analyses showed that levels of both mRNAs of *BIP103* and *BIP131* increased significantly in the lamina joint 2-6 h after brassinolide treatment (**Fig. 4**). The lamina joint cells are very sensitive to BRs and suitable for a bioassay known as lamina inclination test (Abe *et al.*, 1984). Rapid decrease in the level of *OsBRI1* in the experiment indicates that the lamina cells grown in dark responded properly to the exogenous brassinolide as observed by Yamamuro *et al.* (2000). Comparing to *OsBRI1*, the expression levels of *BIP103* and *BIP131* were very low in the absence of brassinolide but increased rapidly to a plateau in 6 h. In contrast to the negative feedback system acting on *OsBRI1* expression, opposite systems may operate for *BIP103* and *BIP131* expressions. The induction of *BIP103* and *BIP131* expressions may have some roles to amplify the BR signal. Currently, we are carrying out *in situ* RNA hybridization experiments to see if the expression of *BIP103* and *BIP131* in addition to other candidate genes will change in the lamina joint tissue treated with brassinolide.

It would be interesting if we could confirm the phosphorylation by BRI1 for *Arabidopsis* PPI. Such comparative experiments in two different plant systems will strengthen the possibility of their regulatory roles. Furthermore, the *in vivo* interaction should be confirmed in these plants to establish the significance of the *in vitro* results. Such issue may be also addressed by introducing RNA interference method to investigate the effects of BIP and PPI gene silencing on the brassinolide responses.

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