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Petunia Germinating Pollen S/D3 Interacts with S-RNases in *Petunia hybrida* Vilm.

Yan-Xia Guo[1,2], Yan-Sheng Zhang[1] and Yong-Biao Xue[1*]

(1. *Laboratory of Molecular and Developmental Biology*, *Institute of Genetics and Developmental Biology*, *the Chinese Academy of Sciences and National Centre for Plant Gene Research*, Beijing 100080, China;
2. *Graduate School of the Chinese Academy of Sciences*, Beijing 100039, China)

Abstract

Self-incompatibility (SI) is a genetic mechanism of self/non-self pollen recognition to prevent self-fertilization in many flowering plants and, in most cases, this is controlled by a multi-allelic *S*-locus. S-RNase and S-locus F box (SLF) proteins have been shown to be the female and male determinants of gametophytic self-incompatibility (GSI), respectively, in the Solanaceae, Scrophulariaceae and Rosaceae. Nevertheless, it is thought that additional factors are required for the SI response. Herein, we constructed a mature anther cDNA library from a self-incompatible *Petunia hybrida* Vilm. line of the S_3S_3 haplotype. Using AhS_2-RNase from *Antirrhinum hispanicum* as a bait for yeast two-hybrid screening, we found that petunia germinating pollen (PGP) S/D3 was capable of interacting physically with the bait. However, the interaction lacked haplotype specificity. The *PGPS/D3* gene is a single copy gene that is expressed in tissues such as the style, ovary, pollen, and leaf. The PGPS/D3::GFP (green fluorescence protein) construct was detected in both the membrane and cytoplasm. The implications of these findings in the operation of S-RNase-based SI are discussed.

Key words: petunia germinating pollen (PGP) S/D3; *Petunia hybrida*; self-incompatibility; S-RNase.

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Self-incompatibility (SI) is a genetic mechanism to prevent self-fertilization in many flowering plants (de Nettancourt 2001). In most species, SI is controlled by a multi-allelic *S*-locus. In species with gametophytic self-incompatibility (GSI), the pollen is rejected when the *S*-haplotype that it expresses matches either of the two *S*-haplotypes expressed in the sporophytic tissue of the pistil (de Nettancourt 2001). In the Solanaceae, Scrophulariaceae and Rosaceae, it has been demonstrated that S-RNase and S-locus F-box (SLF) proteins control stylar and pollen SI, respectively (Lee et al. 1994; Murfett et al. 1994; Qiao et al. 2004a; Sijacic et al. 2004).

Although S-RNase has been shown to be the female determinant of GSI, some additional stylar factors are thought to be required for the SI response (Cruz-Garcia et al. 2003). S-RNase is expressed in cells of the stigma, style, and ovary and is secreted into the extracellular matrix (ECM; Anderson et al. 1986, 1989; Cornish et al. 1987). The S-RNases appeared to enter both self- and non-self-pollen tubes without *S*-haplotype specificity (Luu et al. 2000), but little is known about the mechanism of S-RNase entry into the pollen tube. To understand S-RNase access to the pollen tube cytoplasm, some S-RNase-binding proteins have been isolated from style extracts of *Nicotiana alata*. Of these binding proteins, *N. alata* transmitting tissue-specific glycoprotein (NaTTS), 120 kDa glycoprotein, and *N. alata* pistil extensin-like protein III (NaPELPIII) have been shown to interact with S-RNases *in vitro* (Cruz-Garcia et al. 2005). These glycoproteins have all been shown to also interact with pollen tubes (Wu et al. 1995; Lind et al. 1996; de Graff 1999). However, it remains unclear whether these interactions occur *in vivo*. A small asparagine-rich protein (HT) was isolated from self-incompatible *N. alata*, but was not expressed in self-compatible *N. plumbaginifolia* (McClure et al. 1999). Homologs of HT have been identified from *Lycopersicon* and

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*Author for correspondence. Tel: +86 (0)10 6255 2880; Fax: +86 (0)10 6253 7814; E-mail: <ybxue@genetics.ac.cn>.

Solanum species (Kondo et al. 2002; O'Brien et al. 2002). Two *HT-B*-suppressed transformants of self-incompatible *S. chacoense* expressed S-RNase normally, but did not show *S*-haplotype-specific pollen rejection, indicating that HT-B is required for the SI response, although the exact mechanism is still unclear (O'Brien et al. 2002). Furthermore, a yeast two-hybrid screening system has been used to clone S-RNase-interacting proteins from petunia pollen. *Petunia hybrida* S-RNase-binding protein (PhSBP1) has been found to interact with S-RNase without *S*-haplotype specificity in yeast (Sims and Ordanic 2001). A homolog of PhSBP (ScSBP1) was also identified from *S. chacoense* (O'Brien et al. 2004). However, it remains unknown whether PhSBP1 or ScSBP1 can interact with S-RNase *in vivo* and what role, if any, they play in the SI response.

In order to identify additional factors that are required for the GSI response, we initiated a yeast two-hybrid screen to isolate pollen factors that could interact with S-RNase *in vitro*. Herein, we report the isolation of a protein known as PGP (petunia germinating protein) S/D3 from *P. hybrida* (Virginie et al. 2000) and show that it interacted with with S-RNases in a non-*S*-haplotype-specific manner.

Results

The PGPS/D3 protein interacts with S-RNases without S-haplotype specificity

To isolate pollen factors interacting with AhS_2-RNase, a cDNA library was constructed from mature anthers of self-incompatible *P. hybrida* of the S_3S_3 haplotype. *AhS_2-RNase* lacking the signal peptide sequence was inserted into *$pGBKT_7$* and was used as a bait to screen the cDNA library (Figure 1A). Six million cotransformed yeast cells were plated and, after the final stringent screening, four yeast clones survived on the medium lacking His. Furthermore, these four clones expressed significant β-galactosidase reporter gene activity (data not shown). DNA sequencing analysis revealed that one clone corresponded to PGPS/D3 (Virginie et al. 2000). We focused our initial study on PGPS/D3 because it was shown previously to be upregulated in germinating pollen (Virginie et al. 2000).

To confirm the physical interaction between PGPS/D3 and AhS_2-RNase and detect whether the same interaction also occurs between PGPS/D3 and other S-RNases, we used a yeast two-hybrid assay. In the present study, AhS_2-, S_4-, S_5-, PhS_3-, and Sv-RNases were used as baits and PGPS/D3 was used as the prey. Individual cDNA of S-RNases lacking signal peptide sequences were introduced into the $pGBKT_7$ vector and expressed as a fusion to the GAL4 DNA-binding domain (BD), whereas *PGPS/D3* was introduced into *$pGADT_7$* vector and expressed as a fusion to GAL4 transcriptional activating domain (AD). Five *BD:S-RNases* were transformed into yeast

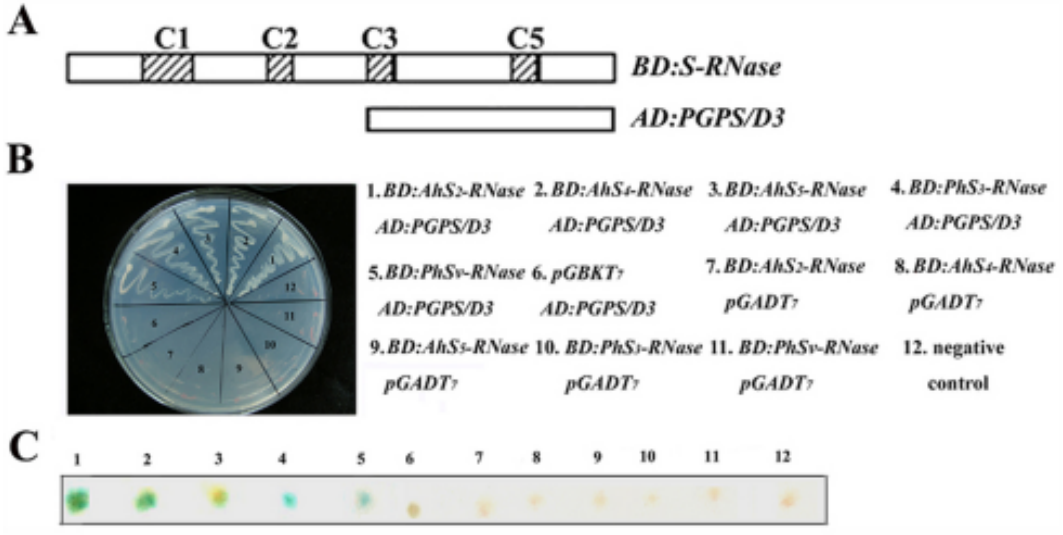

Figure 1. Physical interaction between petunia germinating pollen (PGP) S/D3 and S-RNases.

(A) Schematic diagrams of *BD:S-RNases* and *AD:PGPS/D3* constructs.

(B) Yeast cells containing various combinations of binding domain (BD) and activating domain (AD) fusions were tested for their growth on –Leu/–Trp/–His/–Ade dropout medium. Plasmid $pGBKT_7$ with AD:PGPS/D3, plasmid $pGADT_7$ with various BD:constructs, and plasmid $pGBKT_7$ with $pGADT_7$ were used as negative controls.

(C) Strains were grown further to test for expression of the β-galactosidase reporter gene.

AH109 in combination with *AD:PGPS/D3*. Transformed yeast cells could grow normally on SD/–Leu/–Trp medium and selective SD/–Trp/–Leu/–His/–Ade medium, whereas those transformed with the control plasmids *AD:PGPS/D3* and *pGBKT*$_7$ or *BD:S-RNase* and *pGADT*$_7$ could not grow (Figure 1B), indicating that physical interactions occurred between S-RNases and PGPS/D3 in yeast. Furthermore, β-galactosidase reporter gene activity was detected and appeared to be positive in the yeast cells transformed with *AD:PGPS/D3* and *BD:S-RNases*, but was negative in those transformed with the control plasmids (Figure 1C). These results indicate that PGPS/D3 is able to interact with S-RNases in yeast in a non-*S*-haplotype-specific manner.

Genomic organization of PGPS/D3 in *P. hybrida*

The copy number of the *PGPS/D3* gene was determined by digestion of *P. hybrida* leaf genomic DNA followed by DNA gel blot analysis. A labeled full-length *PGPS/D3* cDNA was used to probe the membrane. A single band of 12, 2.2 and 5.5 kb was detected in genomic DNA digested with *Bam*HI, *Hin*dIII and *Eco*RI, respectively (Figure 2A). Because no restriction sites for three enzymes were detected in the full-length cDNA, this result showed that *PGPS/D3* was present as a single copy in the *P. hybrida* genome. The full-length genomic DNA of PGPS/D3 was obtained from *P. hybrida* genomic DNA using *PGPS/D3* cDNA 5' and 3' primers for PCR (Figure 2B). DNA sequencing analysis revealed that the genomic DNA of PGPS/D3 was 0.82 kb in length and had an intron of 0.48 kb between two exons of 1–35 and 36–121 amino acids (Figure 2C).

Expression pattern and subcellular localization of PGPS/D3

To examine the expression pattern of *PGPS/D3*, RT-PCR was conducted using *PGPS/D3* cDNA-specific primers. The results showed that *PGPS/D3* is expressed in the style, ovary, pollen, and leaf, without any apparent tissue specificity (Figure 3A). Therefore, PGPS/D3 is not a pollen-specific protein. Sequence comparison showed that PGPS/D3 shares a high similarity with neuromodulin, which is a membrane-anchored calmodulin (CaM)-binding protein (Virginie et al. 2000). To examine whether PGPS/D3 is also a membrane-anchored protein, *PGPS/D3* cDNA was introduced into the *pBI221-35S:GFP* vector and expressed as a fusion to green fluorescent protein (GFP). The plasmid *pBI221-35S:GFP-PGPS/D3* was bombarded into onion epidermal cells and green signals in the cells were detected under confocal microscopy. The green signal showed that PGPS/D3 was located in both the membrane and cytoplasm (Figure 3B).

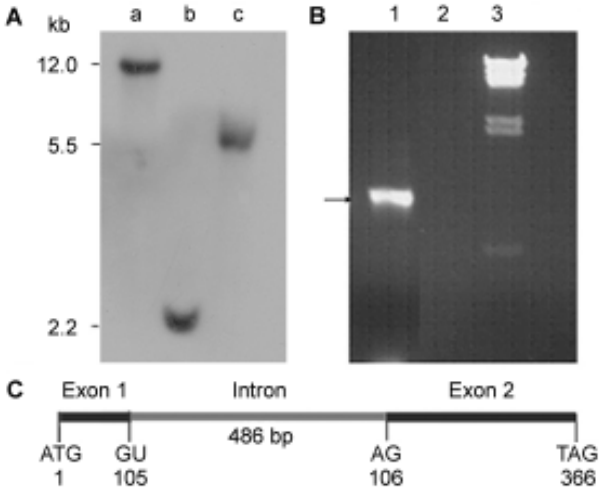

Figure 2. Genomic organization of petunia germinating pollen (PGP) S/D3.

(A) DNA blot analysis of *PGPS/D3*; 10 μg leaf genomic DNA was digested with *Bam*HI (a), *Hin*dIII (b), or *Eco*RI (c) and was blotted and probed with *PGPS/D3* cDNA. Sizes of the hybridizing bands are marked on the left.

(B) A *PGPS/D3* full-length genomic DNA fragment (0.82 kb) was amplified from *Petunia hybrida* leaf genomic DNA. Lane 1, *P. hybrida* leaf genomic DNA was used as template for PCR; lane 2, negative control, with water as a template for PCR; lane 3, DNA molecular weight marker.

(C) Schematic diagram of the full-length *PGPS/D3* genomic DNA. There was an intron of 0.48 kb between exon 1 (1–105) and exon 2 (106–336).

Discussion

Although S-RNase and SLF have been demonstrated to be the female and male determinants of GSI, respectively, little is known about additional factors that are required for the SI response. The results of the present study showed that PGPS/D3 could interact with S-RNases in a non-*S*-haplotype-specific manner in yeast, suggesting a possible role of PGPS/D3 in SI.

It is generally thought that after S-RNase enters pollen tube, it functions as an *S*-haplotype-specific cytotoxin by degrading rRNA in self-pollen tube cytoplasm, leading to the cessation of pollen tube growth in the pistil (McClure et al. 1990; Gray et al. 1991; McCubbin et al. 2000). However, it is unclear whether that is the case *in vivo* and, if so, how S-RNase enters the pollen cytoplasm.

It is unclear how PGPS/D3 could function in SI. Most proteins are taken up into the cytoplasm via endocytosis and many

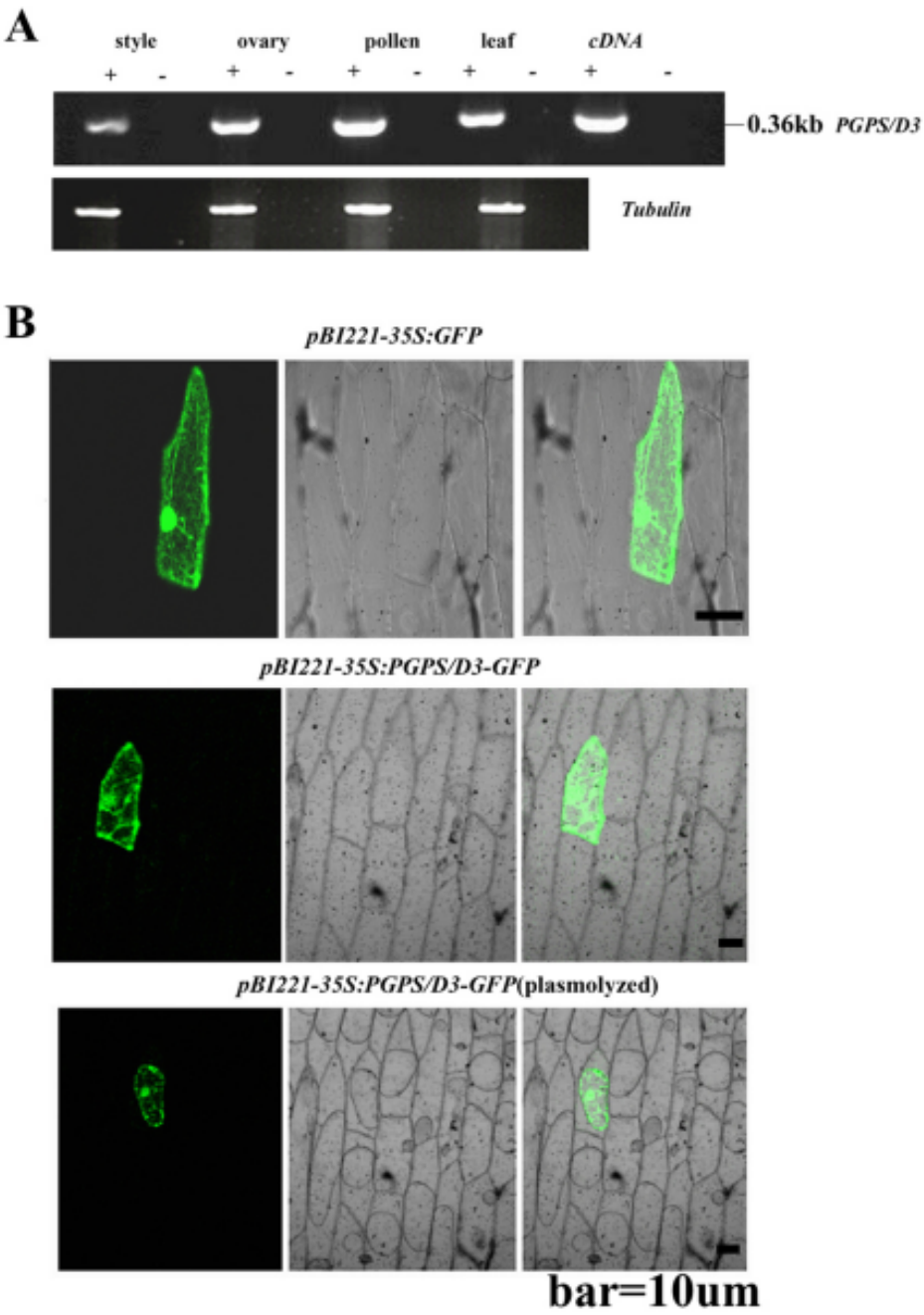

Figure 3. Expression pattern and subcellular localization analyses of petunia germinating pollen (PGP) S/D3.

(A) RT-PCR analysis of RNA from *Petunia hybrida* style, ovary, pollen, and leaf with (+) or without (–) reverse transcriptase in the synthesis of cDNA using specific primers of *PGPS/D3*. The plasmid *AD:PGPS/D3* was used as a positive control (top panel) and RT-PCR analysis of *Tubulin* for loading control is shown in the bottom panel.

(B) It was found that PGPS/D3 localizes to both membrane and cytoplasm. Onion epidermal cells were bombarded with plasmids harboring the green fluorescent protein (GFP) coding region (*pBI221-35S:GFP*) or a PGPS/D3-GFP fusion construct (middle and bottom in normal and plasmolyzed cells, respectively). The PGPS/D3-GFP fusion protein accumulated in the membrane and cytoplasm. Detection of fluorescence was performed 24 h after bombardment under a confocal laser scanning microscope. The right section shows the merged images of those obtained under normal and ultraviolet light.

related factors have been identified. These include some key proteins, such as adaptor protein (AP) 2 (González-Gaitán et al. 1997), AP180 (Ye and Lafer 1995), Epsin (Eps15-interacting protein; Chen et al. 1998), dynamin (van der Bliek et al. 1993; Warnock and Schmid, 1996; Schmid et al. 1998), and amphiphysin (Grabs et al. 1997; Wigge et al. 1997; Wigge and McMahon 1998). In most cases, these proteins are involved in a receptor-mediated endocytosis as a member of an endosome complex. They also have some structural features in common: (i) tyrosine protein kinase phosphorylation site; (ii) binding site for phosphatidylinositol; and (iii) one or more D-Asp, P-Pro, W-Trp (DPW) or DPF (F-Phe) domains (Matthew et al. 1999). The *PGPS/D3* gene was initially isolated as a differentially expressed gene in pollen and sequence analyses showed that it is an acid protein of 121 amino acids with the following characteristics: (i) a potential phosphorylation site for protein kinase C; (ii) a tyrosine kinase phosphorylation site; and (iii) a potential myristylation site in the first seven amino acids at the N-terminal (Virginie et al. 2000). These features suggest that PGPS/D3 may facilitate S-RNase entry into the pollen tube. In the present study, GFP fusion protein localization indicated that PGPS/D3 was localized not only in the plasma membrane, but also in the cytoplasm (Figure 3B). Further examination using immunohistochemity may reveal whether PGPS/D3 is localized in other membrane compartments, such as the endoplasmic reticulum or Golgi membranes.

In the present study, RT-PCR analysis showed that PGPSD/3 appears to be expressed ubiquitously in *P. hybrida*, indicating that apart from its possible role in the SI response, it also likely functions in other physiological processes. Further functional studies on PGPS/D3, as well as other potential S-RNase interacting factors, could provide us with some clues as to the mechanism of S-RNase entry into the pollen tube.

Materials and Methods

Plant materials and cDNA library construction

The SI line of S_3S_3 *Petunia hybrida* Vilm. was described previously (Qiao et al. 2004b). Total RNA was isolated from its mature anther with Trizol (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Poly $A^{(+)}$ RNA was isolated with a MicroPoly(A)Pure kit (Invitrogen). Double-stranded cDNA synthesis, linker ligation, DNA purification, and ligation to vector pGADGH were performed using a cDNA synthesis kit (Stratagen, La Jolla, CA, USA). The cDNA library contains 4×10^6 independent clones with an average insert size of 1.0 kb. The plasmid was isolated using a Qiquick Plasmid Isolation Kit (Qiagen, Valencia, CA, USA).

Construction of Gal4-bait plasmids

AhS_2-RNase, AhS_4-RNase, AhS_5-RNase, PhS_3-RNase and PhS_v-RNase ORFs lacking signal peptides (Qiao et al. 2004b; Robbins et al. 2000) were cloned into $pGBKT_7$ (BD) (Clontech, Palo Alto, CA, USA). Forward primers contained an *Eco*RI or *Pst*I restriction site and a short linker sequence of three alanine or glycine codons prior to the S-RNase sequence. Reverse primers contained an *Sal*I or *Sma*I restriction site. The PCR was performed in a final concentration of 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1 μmol/L primers, and 2.5 U *Taq* polymerase (TaKaRa, Dalian, China) with 10 ng plasmid DNA as the template. Thermocycling conditions were 94 °C for 2 min, then 35 cycles of 94 °C for 1 min, 55 or 58 °C for 1 min, and 72 °C for 1 min, with 72 °C for 10 min in the last cycle. The PCR products were gel purified using a DNA-purification kit (Omega, Doraville, GA, USA), digested with *Eco*RI/*Pst*I and *Sal*I/*Sma*I and ligated into *Eco*RI/*Sal*I- or *Pst*I/*Sma*I digested $pGBKT_7$ (Clontech) to produce chimeric gene constructs in which the Gal4-binding domain region was fused in frame to S-RNase coding sequences. Bait plasmids were transformed into *Escherichia coli* DH5α (Stratagene, La Jolla, CA, USA) and transformants were screened according to the procedure of Beuken et al. (1998). Transformants having the correct insert size were sequenced to verify that they were in frame and that no mutations had been introduced. Bait plasmids were then used to transform yeast strain HF7C (James et al. 1996) by means of LiAc transformation (Clontech).

Yeast two-hybrid assay

Two-hybrid assays were performed with the yeast strain of HF7C, using a sequential transformation procedure in which HF7C was transformed initially with different bait constructs and yeast lines with individual bait plasmids were then transformed with 50 μg DNA isolated from the cDNA library using a large-scale library transformation procedure (Clontech). The yeast mixture was plated on synthetic dropout (SD) medium (Adams et al. 1997) lacking tryptophan, leucine, and histidine (SD/–Trp/–Leu/–His). Positive colonies (containing both BD and AD plasmids and activating *HIS3* reporter gene) were then assayed for β-galactosidase activity (Adams et al. 1997).

Yeast colonies that scored as His+ and β-gal+ in initial screens were used for isolation of activation domain plasmid and re-tested using theyeast two-hybrid assay. Total yeast DNA was isolated (Adams et al. 1997) and used to transform *E. coli* DH5α. Transformants were selected on LB medium with ampicillin, and insert sizes were determined by PCR amplification using Gal4-AD vector primers (Stratagene). Activation domain/cDNA fusion plasmids were then used to transform AH019 (James et al. 1996) together with bait plasmids for the detectino

of Ade+ and β-gal+ activity.

DNA gel blot analysis

Genomic DNA isolation was performed as described previously (Xue et al. 1996).

DNA (10 μg) was digested, separated on a 0.8% agarose gel and transferred onto Hybond N^+ (Amersham, Buckinghamshire, UK) membrane. Prehybridization, hybridization, and washing of the blot were performed as recommended by the manufacturers. Probes were labeled with ^{32}P by random priming using Prime-a-Gene labeling system (Promega, Madison, WI, USA).

RT-PCR analysis

First-strand cDNA was synthesized with 5 μg total RNA or 1 μg poly(A)$^+$ RNA as a template for MMLV reverse transcriptase (Invitrogen). After first-strand synthesis and inactivation of reverse transcriptase, 2 μL cDNA reaction products was used as a template for PCR. Amplifications were performed in a volume of 100 μL in 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 0.2 μmol/L each primer, and 2.5 U *Taq* polymerase (TaKaRa). For control amplification, 10 ng PGPS/D3 cDNA clone was used as a template. Reactions were amplified with the following program: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72°C for 1 min, with 72 °C in the last cycle.

Localization of PGPS/D3-GFP fusion protein

Localization of PGPS/D3 was tested by transient expression of PGPS/D3-GFP fusion protein in onion (*Allium cepa*) epidermal cells. The PGPS/D3 cDNA was amplified with *AD:PGPS/D3* serving as a template. The *pBI221-35S:PGPS/D3-GFP* construct was used for transient transformation of onion epidermal cells using bombardment (Bio-Rad, Hercules, CA, USA). Expression and localization of *pBI221-35S:GFP* (positive control) and *pBI221-35S:PGPS/D3-GFP* were observed under a confocal laser scanning microscope (LSM 510 META; Zeiss, Jena, Germany) using a wavelength of 488 nm. Plasmolysis of onion epidermal cells was induced by 5 mol/L NaCl.

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