Antagonistic Jacalin-Related Lectins Regulate the Size of ER Body-Type β-Glucosidase Complexes in Arabidopsis thaliana

Atsushi J. Nagano 1, Yoichiro Fukao 2, Masayuki Fujiwara 2, Mikio Nishimura 3 and Ikuko Hara-Nishimura 1,*

1 Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, 606-8502 Japan
2 Plant Science Education Unit, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, 630-0101 Japan
3 Department of Cell Biology, National Institute for Basic Biology, Okazaki, Aichi, 444-8585 Japan

Introduction

Glycoside hydrolase family 1, β-glucosidase (BGLU), is widely distributed in prokaryotes and eukaryotes (Xu et al. 2004). One of their major roles in plants is chemical defense (Rask et al. 2000, Lipka et al. 2005). Arabidopsis thaliana has 47 β-glucosidases (Xu et al. 2004), one of which is PYK10/BGLU23 with an endoplasmic reticulum (ER) retention signal (KDEL) (Matsushima et al. 2004, Xu et al. 2004). PYK10 was first reported as a seedling- and mature root-specific β-glucosidase (Nitz et al. 2001). Previously, we reported that PYK10 is localized in ER bodies and has β-D-glucosidase and β-D-fucosidase activities (Matsushima et al. 2004).

ER bodies are ER-derived organelles that may be involved in defense systems (Hayashi et al. 2001, Matsushima et al. 2002, Hara-Nishimura and Matsushima 2003, Matsushima et al. 2003a). Electron micrographs show ribosomes on the surface of ER bodies, which indicates that ER bodies are directly derived from the ER (Hayashi et al. 2001). In transgenic A. thaliana expressing green fluorescent protein (GFP) with an ER retention signal, ER bodies appear as spindle-shaped, GFP-fluorescent structures (~10 μm long and ~1 μm wide) (Haseloff et al. 1997, Ridge et al. 1999, Hawes et al. 2001, Hayashi et al. 2001). ER bodies are observed in seedlings and mature roots. Rosette leaves have few ER bodies. However, wounding rosette leaves or treating them with exogenous methyl jasmonate (a plant hormone that induces a defense response) induces many spindle-shaped structures that resemble ER bodies (Matsushima et al. 2002). These observations suggest that ER bodies have a role in plant defenses against herbivores and/or pathogens. In other Brassicaceae plants, structures like ER bodies were described as dilated cisternae (Bonnett and Newcomb 1965, Iversen 1970, Behnke and Eschlebeck 1978, Bone et al. 1989).

We previously isolated a mutant, nai1-1, that has no ER bodies (Matsushima et al. 2002b). The NAI1 gene encodes a putative basic helix–loop–helix (bHLH)-type transcription factor (Heim et al. 2003, Toledo-Ortiz et al. 2003, Matsushima et al. 2004), and, therefore, is expected to

Keywords: Arabidopsis thaliana — ER body — GDSL lipase-like protein — β-Glucosidase — Jacalin-like lectin — PYK10.

Abbreviations: BGAF, β-glucosidase aggregation factor; BGLU, β-glucosidase; bHLH, basic helix–loop–helix; CBB, Coomassie Brilliant Blue; ER, endoplasmic reticulum; ESP, epitheiospecifier protein; GFP, green fluorescent protein; GLL, GDSL lipase-like protein; JAL, jacalin-related lectin; MBP, myrosinase-binding protein; MS, mass spectrometry; 4-MU, 4-methylumbelliflorone; MyAP, myrosinase-associated protein; PBP1, PYK10-binding protein; RT–PCR, reverse transcription–PCR.

*Corresponding author: E-mail, ihnishi@gr.bot.kyoto-u.ac.jp; Fax, +81-75-753-4142.
regulate directly or indirectly the expression of ER body-related genes. The levels of both PYK10 and PBP1 (PYK10-binding protein; At1g16420) were found to be decreased in nail mutants (Matsushima et al. 2003b, Matsushima et al. 2004). PBP1 consists of two repeated regions, each of which is homologous to the x-chain of jacalin, a carbohydrate-binding protein (lectin) of jackfruit (Artocarpus integrifolia) (19–18% identity, 35–34% similarity). Although PYK10 and PBP1 can interact with each other, PBP1 is not localized in ER bodies but in the cytosol (Nagano et al. 2005). These results implied that PYK10 and PBP1 interact with PYK10 when subcellular structures are destroyed by herbivory, pathogenesis or other processes.

In a plant homogenate, whose environment we assumed to be similar to that in damaged tissues, PYK10 was found to have two forms, an active insoluble form and an inactive soluble form (Nagano et al. 2005). Understanding the biological roles of the two forms of PYK10 and the interactions between PYK10 and PBP1 should provide valuable insights into the plant-specific defense system against pathogens.

Arabidopsis thaliana has another type of β-glucosidase (myrosinases), TGG2, which also form large complexes (Ueda et al. 2006), although TGG2 has a different tissue-specific distribution and subcellular localization from PYK10. Myrosinases are Brassicaceae-specific β-glucosidases, which are responsible for hydrolysis of glucosinolate defense compounds of Brassicaceae (Rask et al. 2000). When the tissue is disrupted, the non-toxic glucosinolates are hydrolyzed by myrosinases into biologically active derivatives, isothiocyanates, thiocyanates, nitriles and epithionitriles (Wittstock and Halkier 2002). Myrosinases interact with myrosinase-binding proteins (MBPs), which have a jacalin-related lectin (JAL) domain, in Brassica napus (Lennan et al. 1990, Falk et al. 1995, Taipalensuu et al. 1997, Geshi and Brandt 1998). Some myrosinases occur in complexes with not only MBPs but also myrosinase-associated proteins (MyAPs), which are members of GDSL lipase-like proteins (GLLs) (Taipalensuu et al. 1996, Akoh et al. 2004). MBPs are necessary for the 62–70 kDa myrosinases to form large complexes (200–1,000 kDa) in oilseed rape, because myrosinases do not form large complexes in plants lacking MBPs (Eriksson et al. 2002).

It has been shown that β-glucosidases form large complexes in oat (Avena sativa) (Nisius 1988, Guis-Mayer et al. 1994), maize (Zea mays) (Esen and Blanchard 2000) and flax (Linum usitatissimum) (Fieldes and Gerhardt 1994). In some strains of Zea mays, Glu1 β-glucosidase occurs as part of a large, insoluble complex. BGAF (β-glucosidase aggregation factor) has been shown to bind Glu1 and to be responsible for the formation of the complex (Esen and Blanchard 2000, Blanchard et al. 2001). BGAF has a JAL domain. A careful biochemical study of BGAF revealed that the JAL domain of BGAF is responsible for the polymerization of Glu1 (Kittur et al. 2007). The active PYK10 complex is similar to the myrosinase complex in Brassicaceae plants, PYK10 corresponds to myrosinase, while PBP1 and JALs correspond to MBPs, and GLLs correspond to MyAPs.

The biological significance of the interactions between β-glucosidases and their partners are not known. To understand how β-glucosidases and its related proteins mediate defense against pathogens, we characterized an active insoluble form of PYK10 and investigated ER body-related genes. In this study, we report the components of the PYK10 complex. We also report that two types of JALs having opposite roles regulate the size of the PYK10 complex antagonistically.

Results

Genome-wide survey of JAL and GLL genes

The myrosinases in Brassica form complexes containing some JAL and GLL proteins (Taipalensuu et al. 1996). PBP1, an interactant of PYK10, is a JAL (Nagano et al. 2005). These results suggest that JAL and GLL are associated with PYK10 β-glucosidase. A search of the A. thaliana genome database found 48 JAL genes (Figs. 1, 2) and 109 GLL genes (Supplementary Fig. S1 and Table S2). The JAL and GLL genes were named according to their locations on the chromosomes. Some of the JAL and GLL genes form clusters on the chromosomes (Supplementary Fig. S2). The subcellular localizations of JAL and GLL proteins were predicted with the Wolf-PSORT and TargetP programs. It was predicted that a large part of the JAL proteins are localized in the cytosol, and that a large part of the GLL proteins are localized in the extracellular matrix or vacuole (Supplementary Tables S1, S2).

The JAL proteins of Arabidopsis and other plants have different combinations of seven different types of JAL domains (Figs. 1, 2). Six JAL proteins (Nos. 21, 27, 28, 29, 37 and 38) are chimeric. JAL37 and JAL38 have an F-box-associated domain only once. JAL21, JAL27, JAL28 and JAL29 are chimeric lectins with several kelch repeats. The JAL domains adjacent to the kelch repeats made a monophyletic group (dark green region). This shows that the chimerization between the JAL domain and the kelch repeat occurred only once. JAL21, JAL27, JAL28 and JAL29 are chimeric lectins with several kelch repeats. The JAL domains adjacent to the kelch repeats made a monophyletic group (dark green region). This shows that the chimerization between the JAL domain and the kelch repeat occurred only once.

PYK10 is co-expressed with some JAL and GLL genes

A survey of the Arabidopsis genome revealed 48 JAL genes and 109 GLL genes. To determine which JAL
and GLL proteins work in concert with PYK10, we applied three different approaches: co-expression analysis, comparative transcriptomics and biochemical analysis of the isolated PYK10 complex.

We postulate that JAL and GLL proteins that interact with PYK10 should be expressed in the same tissues and at the same developmental stages as PYK10. To identify JAL and GLL proteins that are co-expressed with PYK10, hierarchical clustering analysis was performed using a public DNA microarray data set of various developmental stages (Supplementary Fig. S3). Some clusters were identified from the result of hierarchical clustering analysis (Supplementary Fig. S3, right side). We identified 30, 14 and 10 gene clusters at distance thresholds of 20, 25 and 30, respectively. The cluster containing PYK10 was conserved whenever the distance threshold was lower than 20.

Fig. 1 Neighbor-joining distance tree of A. thaliana and other plant JAL domains. The amino acid sequences were aligned by Clustal W. JAL domains of A. thaliana are indicated by their AGI codes and their order in the JAL domain repeat. For example, At5g46000.1 JR 1/3 shows the first JAL domain in three repeated domains of At5g46000.1 protein. JALs of other plant species and their GenBank accession numbers are shown below. Jacalin α-chain (P18670), MPA (P18674), BGAF (AAF71261), BnMBP1 (CAA70587), BnMBP2 (AAC08048), BnMBP3 (AAC08049), BnMBP4 (AAC08050), BnMBP5 (CAA72270), BnMBP6 (CAA72271) and BnMBPRP (AAC08051). Nictaba (AAK84134), which is not a JAL but a legume lectin, is used for the outgroup. Colored nodes indicate subgroups of JAL domains. Names of genes dealt with in this study are in red.
threshold was set at 20, 25 and 30, indicating that it was well isolated from other clusters (Supplementary Fig. S3, red boxes). Other members of this cluster were JAL22, JAL31, JAL33, JAL34 and GLL23. These results raise the possibility that certain JAL and GLL proteins are the interaction partners of PYK10.

**mRNA levels of some JAL and GLL genes are reduced in the nai1-1 mutant**

To identify genes that are related to PYK10 and ER bodies, we compared the genome-wide expression profiles of Col-0 and the nai1-1 mutant using DNA microarrays. The expression levels of 29 genes were 2-fold higher in the nai1-1 mutant than in Col-0, and the expression levels of 341 genes were 2-fold lower in the nai1-1 mutant than in Col-0. Five JAL genes (JAL22, JAL23, JAL31, JAL33 and PBP1/JAL30) were found in the best 15 genes showing low levels of expression in nai1-1 (Table 1). The mRNA level of PYK10 was reduced in nai1-1 (4.3-fold larger in Col-0 than in nai1-1). The mRNA levels of GLL23 and GLL25 were also reduced in nai1-1 (3.1- and 3.0-fold). Those genes might be transcriptionally regulated directly or indirectly by NAI1, because NAI1 is a bHLH-type transcriptional factor.

**Active PYK10 molecules form micrometer-sized complexes**

We used 4-methylumbelliferone (4-MU) β-D-glucopyranoside as a substrate to measure the β-D-glucosidase activity of PYK10. The activity relative to total protein in the crude extract was 91 pmol 4-MU s⁻¹ mg⁻¹ total protein (546 pmol 4-MU min⁻¹ mg⁻¹ total protein) (Fig. 3C). The activity of PYK10 was estimated to be 0.35 μmol 4-MU min⁻¹ mg⁻¹ active PYK10 protein, considering that the proportion of total PYK10 protein to total protein was approximately 3% (Fig. 4A), that the proportion of active PYK10 to total PYK10 was at most one-third (Fig. 3B) and that the proportion of the activity contributed by PYK10 to β-D-glucosidase activity was 65% (see below). The estimated specific activity of 0.35 μmol 4-MU min⁻¹ mg⁻¹ is enough for PYK10 to function.

PYK10 has two forms: an insoluble active form and a soluble inactive form. We investigated the size of the active PYK10 complex using differential centrifugation (Fig. 3A, B, Supplementary Fig. S5) and filter treatments (Fig. 3C). Before incubation of the extracts from Arabidopsis roots, the highest activity was observed in the S15 fraction (Fig. 3A, 0 h). After incubation, the highest activity was observed in the P15 fraction (Fig. 3A, 24 h). The activities in
the P15 and S15 fractions changed dramatically before and after incubation (\(P<0.01\), Welch's t-test). These results indicate that the size of the active PYK10 complex increases during incubation. To elucidate the relationship between PYK10 activity and the amount of PYK10 protein, the fractions whose activities were measured were subjected to immunoblot analysis with anti-PYK10 antibodies. In the P1, P4 and P15 fractions, PYK10 activities seemed to be correlated with the amount of PYK10 protein. However, the S15 fraction had a large amount of PYK10 protein, although its activity was low (Fig. 3A, S15 of 24 h). These results are consistent with PYK10 having two forms. The activity of root homogenates was reduced about 20% by passing them through a 70 m\(\mu\)m pore filter, was reduced about 60% by passing them through a 5 m\(\mu\)m pore filter and was undetectable after passing them through a 0.65 m\(\mu\)m pore filter (Fig. 3C). These results showed that the size of active PYK10 complex ranged from 0.65 to 470 m\(\mu\)m. Some mono-saccharides inhibited the complex formation (Supplementary Fig. S6). This result suggests that an interaction between lectin and carbohydrate is involved in the complex formation.

Identification of members of the active PYK10 complex

The P15 fraction, in which the PYK10 complex is abundant (Fig. 4A), was subjected to sucrose density gradient centrifugation to remove contaminating proteins. The active PYK10 complex had a specific density (Fig. 4B, fraction 1). Fraction 13 of the sucrose density gradient was concentrated by ultrafiltration and subjected to SDS–PAGE (Fig. 4C). We identified five JAL and 11 other proteins by tandem mass spectroscopy (MS/MS) analysis (Fig. 4C and Table 2). The transcriptional levels of six of the identified proteins (JAL34, PYK10, PBP1, JAL31, JAL33 and At5g26280) were 2-fold lower in nai1-1 than in Col-0.

The pyk10-1 mutant has a T-DNA that was inserted in the first intron of the PYK10 gene (Supplementary Fig. S8). PYK10 was not detected in pyk10-1 by immunoblot analysis with anti-PYK10 antibodies (Fig. 4A). However, pyk10-1 has the \(\beta\)-glucosidase activity, which was 35% of that in Col-0. pyk10-1 gave a concentrated protein complex in fraction 13 of the sucrose density gradient (Fig. 4B), although the amount of protein was much lower than that in the corresponding fractions 12 and 13 of Col-0 (Fig. 4C). BGLU21 and BGLU22 were identified in the complex of pyk10-1 (Fig. 4C and Table 2). Neither BGLU21 nor BGLU22 was identified in the complex of Col-0, probably because the amount of PYK10 is too high to detect minor amounts of BGLU21 and BGLU22. The results suggest that the PYK10 complex of Col-0 contains BGLU21 and BGLU22.

Isolation of jal and gll mutants

The DNA microarray analysis of the nai1-1 mutant root revealed that the mRNA levels of some JAL and GLL genes (JAL22, JAL23, JAL31, JAL33, PBP1/JAL30, GLL23 and GLL25) decreased. The hierarchical clustering
with a fluorescent substrate, 4-MU
[BGLU21, BGLU22, PBP1/JAL30 (and/or JAL31), JAL33,
complex revealed that the complex contained many proteins
purification and MS/MS analysis of the active PYK10
expressed in the same developmental stages as PYK10. The
purification and MS/MS analysis of the active PYK10
complex revealed that the complex contained many proteins
[BGLU21, BGLU22, PBP1/JAL30 (and/or JAL31), JAL33,
JAL34, JAL35 and GLL22]. From these results, we
obtained nine candidate genes that associate with PYK10
(JAL22, JAL23, JAL31, JAL33, JAL34, PBPI/JAL30,
GLL22, GLL23 and GLL25).

To determine the functions of the candidate genes in
the active PYK10 complex, we applied reverse genetics.
Homozygous T-DNA insertion mutants of the candid-
ate genes were identified by PCR-based genotyping. The
T-DNA insertion sites in each mutant were confirmed by
direct sequencing (Supplementary Fig. S8). The mRNA
levels of the disrupted genes were checked by reverse
transcription–PCR (RT–PCR) to confirm that the genes
were not expressed. The mRNA levels of T-DNA inserted
genes of the mutants (jal22-1, jal22-2, jal23-1, jal31-1,
jal31-2, jal33-1, gl22-1, gl23-1 and gl25-1) were lower than
those of the wild type (Fig. 5). The weak bands observed in
jal31-1 and jal31-2 mutants were due to mRNA of PBP1/
JAL30. No reduction of mRNA levels of the JAL34 gene
was observed in the jal34-1 mutant. The sequence of the
PCR product observed in jal34-1 confirmed that the PCR
product was a proper transcript of the JAL34 gene. Based
on these results, we omitted the jal34-1 mutant from the
following analysis. The mRNA levels of four of the JAL
genes (JAL22, JAL31, JAL33 and JAL34) and three of the
GLL genes (GLL22, GLL23 and GLL25) were lower in the
nai1-1 and nai1-2 mutants than in the wild types. mRNA
levels of JAL genes (JAL22, JAL23, JAL30, JAL31, JAL33
and JAL34) and GLL genes (GLL22, GLL23 and GLL25)
were the same in the pyk10-1 mutant and the wild type
(Supplementary Fig. S7).

Fig. 3  The active PYK10 molecule forms a micrometer-sized
complex. (A) The β-D-glucosidase activities in differential centri-
fugation fractions. The root homogenate from 20-day-old Col-0
was incubated for 0 or 24 h, and then centrifuged sequentially at
1,000 × g (P1), 4,000 × g (P4) and 15,000 × g (P15) at 22 °C
for 3 min. The supernatant after centrifugation at 15,000
was used as the soluble fraction (S15). β-D-Glucosidase activity
measured with a fluorescent substrate, 4-MU β-D-glucopyranoside.
The experiments were done 15 times independently. The average
activities in each fraction before and after incubation are indicated
by diamonds and squares, respectively. In P15 and S15 fractions,
activities at 0 and 24 h were significantly different (P < 0.01,
Welch’s t-test). Error bars indicate the SD (n = 15). (B) In the
insoluble fractions (P1, P4 and P15), the protein amounts of
PYK10 correlate with β-D-glucosidase activities. Each fraction
whose activity was measured was subjected to SDS–PAGE
and immunoblot analysis with anti-PYK10 antibodies. (C)
Activities of root homogenates after passing through filters of the
indicated pore sizes. Bars indicate means of four independent
experiments. Error bars indicate the SD (n = 4).

Sizes of active PYK10 complexes are regulated by JALs

The activities of PYK10 and the homologs (BGLU21
and BGLU22) in total extracts were not different between
Col-0 and the jal and gll mutants (Fig. 6A), indicating
that the candidate genes did not affect the activities of PYK10
and the homologs. Before incubation, the fraction with the
highest PYK10 activity was the S15 fraction in jal23-1,
jal31-1, jal31-2, gl22-1, gl23-1 and gl25-1 (as in Col-0) and
the P4 fraction in pbp1, jal22-1, jal22-2 and jal33-1 (Fig. 6B,
left). However, the activities in the P1 and P4 fractions of
these mutants were not significantly different from that of
Col-0 (Fig. 6B, right; P > 0.05, Welch’s t-test). On the other
hand, after incubation, the fraction with the highest
activities of PYK10 and the homologs were the P15 fraction
in jal23-1, jal31-1, jal31-2 and jal33-1 (as in Col-0) and
the P4 fraction in pbp1, jal22-1, jal22-2, jal33-1, gl22-1,
gl23-1 and gl25-1 (Fig. 6C, left). The activities in the P1 and P4
fractions of Col-0 were significantly different from those of
pbp1-2, jal22-1, jal23-1, jal31-1 and jal31-2 (Fig. 6C, right).
The PYK10 complexes in pbp1-2 and jal22-1 were
significantly larger than those of Col-0 (P < 0.01 and
<0.05, respectively; Welch’s t-test), while the PYK10
complexes in jal23-1, jal31-1 and jal31-2 were significantly smaller than those of Col-0 (P < 0.01). The results suggest that some JALs have opposite roles in size regulation of the PYK10 complex.

Discussion

JAL proteins consist of various numbers of JAL repeats (Figs. 1, 2). Some JAL proteins are chimeric lectins with an F-box domain, an F-box-associated domain or kelch repeats (Figs. 1, 2). The kelch repeat of four chimeric JALs (JAL21, JAL27, JAL28 and JAL29) has high homology to epithiospecifier protein (ESP) (56% identity, 70% similarity). ESP plays a role in the myrosinase–glucosinolate system. The products of glucosinolate hydrolysis are specified from isothiocyanate to nitriles and epithionitriles by ESP (Lambrix et al. 2001). Thus, the four chimeric JALs (JAL21, JAL27, JAL28 and JAL29) may play an ESP-like role in myrosinase–glucosinolate and/or other β-glucosidase–substrate systems. Although little is known about the functions of GLL proteins, a GLL protein also plays a role in the myrosinase–glucosinolate system.
Recently, GLL65 was reported to be an epithiospecifier modifier 1 (ESM1) (Zhang et al. 2006). ESM1 represses nitrile formation and favors isothiocyanate production in glucosinolate hydrolysis by myrosinases (TGG1 and TGG2). The GLLs that were investigated in this study (GLL22, GLL23 and GLL25) are close relatives of ESM1 (Supplementary Fig. S1). This relationship raises the possibility that the GLLs have ESM1-like activities.

TGG1, TGG2 and ESM1 are expressed in leaf, whereas PYK10 and GLL23 are expressed mainly in root (Supplementary Fig. S3) (Ueda et al. 2006). This suggests that GLL23 plays a role in hydrolysis of the unknown substrates by PYK10 in roots as ESM1 plays a role in glucosinolate hydrolysis by TGGs in leaves. GLL25 may play a role in hydrolysis both by PYK10 and by TGGs, because GLL25 is expressed in both leaves and roots. The expression profile of GLL22 during development is not known.

The major protein of the PYK10 complex in the insoluble fraction (P1, P4 and P15) was PYK10 (Fig. 4). Analysis of the pyk10-1 mutant revealed that 65% of β-glucosidase activity was due to PYK10. The remaining activity in pyk10-1 was probably due to BGLU21 and BGLU22, because these proteins were also contained in the PYK10 complex (Fig. 4 and Table 2). BGLU21 and BGLU22 proteins are the closest homologs of PYK10 (Xu et al. 2004) and have a signal peptide at the N-terminus and

<table>
<thead>
<tr>
<th>Band no.</th>
<th>AGI code</th>
<th>Gene name</th>
<th>Description</th>
<th>Fold change (Col-0/nai1-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>At4g24190</td>
<td>SHEPHERD</td>
<td>ER type HSP90</td>
<td>0.68</td>
</tr>
<tr>
<td>2</td>
<td>At3g16460</td>
<td>JAL34</td>
<td>Jacalin-related lectin</td>
<td>3.30</td>
</tr>
<tr>
<td>3</td>
<td>At3g09260</td>
<td>PYK10/BGLU23</td>
<td>Glucosyl hydrolase family 1, β-glucosidase</td>
<td>4.31</td>
</tr>
<tr>
<td>4</td>
<td>At5g08670</td>
<td>ATP synthase α chain</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and/or At5g08680</td>
<td></td>
<td>ATP synthase α chain</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>and/or At5g08690</td>
<td></td>
<td>ATP synthase α chain</td>
<td>0.95</td>
</tr>
<tr>
<td>5</td>
<td>At3g16470</td>
<td>JAL35</td>
<td>Jacalin-related lectin</td>
<td>0.80</td>
</tr>
<tr>
<td>6</td>
<td>At1g58270</td>
<td>ZW9</td>
<td>Meprin and TRAF homology-containing protein</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>and At5g07440</td>
<td></td>
<td>Glutamate hydrogenase</td>
<td>0.96</td>
</tr>
<tr>
<td>7, 8</td>
<td>At1g54000</td>
<td>GLL22</td>
<td>GDSL lipase-like protein</td>
<td>2.36</td>
</tr>
<tr>
<td>9, 10</td>
<td>Not identified</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>At3g16420</td>
<td>PBP1/JAL30</td>
<td>Jacalin-related lectin</td>
<td>6.31</td>
</tr>
<tr>
<td></td>
<td>and/or At3g16430</td>
<td></td>
<td>Jacalin-related lectin</td>
<td>13.50</td>
</tr>
<tr>
<td>12</td>
<td>At3g16450</td>
<td>JAL33</td>
<td>Jacalin-related lectin</td>
<td>22.35</td>
</tr>
<tr>
<td>13</td>
<td>At5g26280</td>
<td>JAL33</td>
<td>Meprin and TRAF homology-containing protein</td>
<td>2.33</td>
</tr>
<tr>
<td>14</td>
<td>At1g66270</td>
<td>BGLU21</td>
<td>Glucosyl hydrolase family 1</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>At1g66280</td>
<td>BGLU22</td>
<td>Glucosyl hydrolase family 1</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Fig. 5 RT–PCR analysis of nai1, jal and gll mutants. Gene names are shown on the left. PCR cycle numbers are shown on the right. Locations of PCR primers and T-DNA insertions are given in Supplementary Fig. S8. PCR primer sequences are described in Table S5. The RNA that was used for reverse transcription was extracted from roots of 20-day-old plants.

Table 2 Proteins identified in sucrose density fraction 13 by MS/MS analysis
a putative ER retention signal at the C-terminus (RDEL and KDEL, respectively). These characteristics suggest that BGLU21 and BGLU22 are also localized in ER bodies.

The PYK10, BGLU21 and BGLU22 proteins formed large complexes with diameters ranging from 0.65 to >70 μm (Fig. 3). The PYK10 complex contained JALs and other proteins (Fig. 4 and Table 2). The stoichiometry and the manner of interaction of the components in the PYK10 complex remain unclear. To clarify the questions, in vitro experiments of complex formation using the components expressed in a heterologous host are needed.

We found that the PYK10 complex was larger in the pbp1-1 and jal22-1 mutants and smaller in the jal23-1, jal31-1 and jal31-2 mutants (Fig. 6). This finding shows that two types of JALs having opposite roles regulate the size of the PYK10 complex antagonistically. We define them as a ‘polymerizer-type lectin’ and an ‘inhibitor-type lectin’: PBP1/JAL30 and JAL22 proteins are inhibitor-type lectins, while JAL31 and JAL23 proteins are polymerizer-type lectins. It is possible that polymerizer-type lectins act as divalent or multivalent lectins to form the large insoluble complex via linking PYK10 and the homologs (BGLU21 and BGLU22), whereas inhibitor-type lectins act as monovalent lectins that have no ability to link the β-glucosidases.

Interestingly, the closest homologs of polymerizer-type lectins (JAL31 and JAL23) were inhibitor-type lectins (PBP1/JAL30 and JAL22) (Figs. 1, 2). This suggests that the function of lectins converted from one type to another type after the duplications of these genes. A pair of homologs having opposite functions, such as the two JALs, is known in key controllers of flowering, FT as a flowering activator and TFL1 as a flowering repressor (Hanzawa et al. 2005). Gene duplication is thought to be the primary source for evolving genes having new function (Ohno 1970). The evolution of genes having a new function from duplicated genes is designated as neofunctionalization. The proportion of neofunctionalization to all duplication events is very small (Lynch and Conery 2000). In spite of the rarity of neofunctionalization, it appears to have occurred twice (and independently) in the conversions from polymerizer-type to inhibitor-type in JAL22–JAL23 and PBP1/JAL30–JAL31 (Figs. 1, 2). The pairs of polymerizer-type and inhibitor-type lectins reported here are good examples of neofunctionalization.

Fraction of P1 and P4 are derived from differential centrifugation. The root homogenate was incubated for 0 h (B) or 24 h (C), and then centrifuged sequentially at 1,000 × g (P1), 4,000 × g (P4) and 15,000 × g (P15) at 22°C for 3 min. The supernatant after centrifugation at 15,000 × g was used as the soluble fraction (S15). Bar graphs (right) show the relative activities of P1 and P4 fractions. Mutants significantly different from Col-0 are shown by an asterisk (*P<0.05, **P<0.01). Activities are expressed relative to total activities. Values indicate the average of more than four experiments. Error bars are the SD.
The activities of PYK10 and the homologs in the \textit{pbp1-1} mutant was reported to be half of that in the wild type (Nagano et al. 2005). In contrast, here we found that the activities of PYK10 and the homologs in \textit{pbp1-1} were not different from that in the wild type. The discrepancy was due to the use of a 5 µm filter for removing debris in our previous study. Because the \textit{pbp1-1} mutant has a larger PYK10 complex than the wild type, larger proportions of PYK10 and the homologs of the \textit{pbp1-1} mutant were removed by filtration with a 5 µm filter (Fig. 6 and Supplementary Fig. S9).

The subcellular localization of PBP1 is different from that of PYK10 (Nagano et al. 2005). The subcellular localizations of JALs and GLLs (except GLL25) studied in Fig. 5 are probably different from that of PYK10. WolfPSORT predicts that such JALs are located in the cytoplasm or cytoskeleton (Supplementary Table S1) and that GLL22 and GLL23 are located in the extracellular matrix (Supplementary Table S2). These findings suggest that PYK10 encounters such JALs and GLLs only when tissue is damaged and the subcellular structures are destroyed, i.e. that it works in damaged tissues. Formation of PYK10 complexes may be a way to shield active PYK10 molecules from inhibitors, proteases and other proteins that reduce the efficiency of PYK10 as a defense system. Nitrile-specifier protein (NSP) in cabbage white butterfly (\textit{Pieris rapae}) was found to redirect glucosinolate hydrolysis, causing it to form a less toxic nitrile instead of a highly toxic isothiocyanate (Wittstock et al. 2004). A protein complex of oat was found to be stable against proteases (Gus-Mayer et al. 1994). On the other hand, the formation of large PYK10 complexes may be a way to shield active PYK10 from inhibitors, proteases and other proteins that destroy, i.e. that it works in damaged tissues. Formation of large complexes means a reduction of the number of enzyme molecules from inhibitors, proteases and other proteins that decrease the efficiency of PYK10 as an enzyme. Therefore, the formation of a large PYK10 complex seems to decrease the efficiency of PYK10 as an enzyme. There is a trade-off between protecting active PYK10 from inhibitors and/or proteases and maintaining the enzymatic efficiency of PYK10. The formation of active PYK10 complex and size regulation of the complex by JALs may be mechanisms for coping with conflicting goals: protection from inhibitors and maintaining enzymatic efficiency.

Materials and Methods

\textbf{Plant materials and growth conditions}

\textit{Arabidopsis thaliana} ecotype Columbia-0 (Col-0) and Wassilewskija (WS) were used as wild-type plants. The \textit{nai1-1} mutant was derived from \textit{nai1-1 GFP-h} (Matsushima et al. 2003b, Matsushima et al. 2004, Nagano et al. 2005). The \textit{nai1-2} mutant (Matsushima et al. 2004) and \textit{pbp1} mutant (Nagano et al. 2005) were previously reported. Mutant lines were isolated from bulked \textit{F_2} or \textit{F_3} seeds of T-DNA insertion lines by PCR-based genotyping (jal22-1 from salk\_018741, jal22-2 from salk\_019269, jal22-3 from salk\_109217, jal31-1 from salk\_026623, jal31-2 from salk\_058053, jal33-1 from salk\_085183, jal34-1 from GABI\_164E10, gll22-1 from salk\_008952, gll23-1 from salk\_05117 and gll25-1 from salk\_135215) (Alonso et al. 2003) or immunoblot analysis with anti-PYK10 antibodies (pyk10-1 from WiscDsLox461–464F2). Gene-specific PCR primers are described in Supplementary Table S3. Seeds were surface sterilized and then sown onto 0.5% (w/v) Gellan Gum (Wako, Tokyo, Japan) with MS medium (Wako) and 1% (w/v) sucrose, and were grown at 22°C under continuous light conditions.

\textbf{RT–PCR analysis}

Total RNA was isolated from root of 20-day-old plants using an RNeasy plant mini kit (Qiagen, Valencia, CA, USA). A 200 ng aliquot of total RNA was subjected to first-strand cDNA synthesis using Ready-To-Go RT-PCR beads (Amersham Bioscience, Piscataway, NJ, USA). PCR conditions were as follows: 94°C for 2 min; 25–40 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 1 min; and a final elongation step of 7 min at 72°C. The gene-specific primers used for PCR are described in Supplementary Table S4. The PCR products were separated by 1.0% agarose gel electrophoresis.

\textbf{Differential centrifugation and filter treatment}

The method of sample preparation and the details of differential centrifugation experiments are described as a flow chart of the protocols in Supplementary Fig. S4. The protein amounts and the \(\beta\)-glucosidase activities of each fraction were measured as previously described (Nagano et al. 2005). Each fraction was subjected to SDS-PAGE followed by either Coomassie Brilliant Blue (CBB) staining or immunoblot analysis as previously described (Nagano et al. 2005). Statistical analysis was performed by R (http://www.R-project.org/). The homogenate of 20-day-old Col-0 root was subjected to filtration with various pore sizes [75 µm Cell strainer (BD Bioscience, Bedford, MA, USA), 5 µm Ultrafree-CL (Millipore, Bedford, MA, USA), 0.65 µm Ultrafree-MC (Millipore)]. Activity measurement was carried out on the flow-through fractions.

\textbf{Genome-wide survey and co-expression analysis of JALs and GLLs}

Lists of JALs and GLLs were made using annotations of the TAIR6 and 5 genome release. Genes having a jacalin-related lectin domain (IPR001229) were designated as JAL genes. Genes having a lipolytic enzyme, G-D-S-L domain (IPR001360) were designated as GLL genes. Subcellular localization was predicted with TargetP (Emanuelsson et al. 2000) and WolfPSORT (Horton et al. 2006). To make neighbor-joining distance trees, the amino acid sequence were aligned by ClustalW (Thompson et al. 1994). The trees were drawn with TreeView (Page 1996). We used expression data of AtGenExpress (development) (Schmid et al. 2005) for the co-expression analysis. Averages of triplicate experiments were used for analysis. Hierarchical clustering and resampling analysis were performed with TIGR MultiExperiment Viewer v3.1 (http://www.tm4.org/mev.html). Pearson correlation was used as a distance metric in hierarchical clustering. The jack-knife resampling method was used in resampling analysis (1,000 iterations).

\textbf{DNA microarray analysis}

Total RNA was isolated from three roots of 20-day-old Col-0 and \textit{nai1-1} using an RNeasy plant mini kit (Qiagen). RNA quality was checked with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A 5 µg aliquot of total RNA was fluorescently labeled using a Low Input RNA Fluorescent Linear Amplification Kit (Agilent Technologies). A 1 µg aliquot of labeled RNA was

---

Downloaded from https://academic.oup.com/pcp/article-abstract/49/6/969/1812486 by guest on 16 April 2019
hybridized to an Arabidopsis 2 DNA microarray slide (Agilent Technologies) using in situ Hybridization kit Plus (Agilent Technologies). The DNA microarray slide was scanned by DNA microarray scanner (Agilent Technologies). Spot intensities on the DNA microarray were quantified by Feature Extraction Software version 7.1.1 (Agilent Technologies) with default parameters. Flagged spots were removed from the subsequent analysis. Biological duplicate experiments and dye-swap experiments were performed.

Purification and MS/MS analysis of the active PYK10 complex

Roots from twenty-four 20-day-old plants were homogenized in 2 ml of 50 mM sodium phosphate (pH 7.0) with an ice-cold mortar and pestle. The homogenate was passed through a 75 µm Cell strainer (BD Bioscience) to remove debris. The flow-through fraction was subjected to filtration using a 5 µm Ultrafree-CL (Millipore). The flow-through fraction was incubated at 22°C for 14 h. After incubation, the flow-through was centrifuged at 15,000 x g at 22°C for 3 min. The precipitate was designated as partially purified active PYK10 complex. The partially purified active PYK10 complex was resuspended by 200 µl of 50 mM sodium phosphate (pH 7.0) and centrifuged in a 30–60% (w/v) sucrose density gradient prepared with Gradient master (Towa Labo, Tokyo, Japan). The samples were centrifuged in an SW28.1 rotor (Beckman, Palo Alto, CA, USA) at 25,000 r.p.m. for 2.5 h at 4°C. Fractions (1 ml each) were collected with a piston gradient fractionator (Towa Labo). A 20 µl aliquot of each fraction was subjected to SDS-PAGE and silver staining. After fraction 1 was denatured in SDS sample buffer, it was concentrated 10-fold by an Ultrafree-MC 5000 NMWL (5000 NMWL, Millipore). The concentrated sample was subjected to SDS-PAGE and CBB staining. Bands were excised from the CBB-stained gel, and were destained with destaining buffer [30% (v/v) acetonitrile and 25 mM ammonium bicarbonate] for 15 min. After alkylation with 0.25 µg of trypsin (Promega, Tokyo, Japan) in 50 µl of 25 mM ammonium bicarbonate, for 16 h at 37°C. Peptides were extracted from gel slices by 30 µl of 5% (v/v) formic acid and 20% (v/v) acetonitrile for 30 min twice, and the solution of extracted peptides was dried using an evaporator. The dried sample was reconstituted by adding 12 µl of 5% formic acid and 5% acetonitrile, and gently pipetting up and down to dissolve the extracted peptides. Trypsin-digested peptides were loaded on the column (PepMapC18, 5 µm, 75 µm internal diameter, 15 cm; Dionex) using the CapLC system (Waters). Buffers were 0.1% HCOOH in 5% acetonitrile (A) and 0.1% HCOOH in 95% acetonitrile (B). A linear gradient from 5 to 45% of B for 25 min was applied, and peptides eluted from the column were introduced directly into a Q-TOF mass spectrometer (Waters) with a flow rate of 100 nI min⁻¹. Ionization was performed with a PicoTip nanospray source (New Objective). MS/MS spectra were subjected to the Mascot server against a protein database from the National Center for Biotechnology Information.

Supplementary material

Supplementary material mentioned in the article is available online at the journal website www.spp.ccpj.oxfordjournals.org.

Funding

Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan Core Research for Evolitional Science and Technology of the Japan Science and Technology Corporation, Grants-in-Aid for Scientific Research (Nos. 16085203 and 17107002); the Global Center of Excellence Program ‘Formation of a Strategic Base for Biodiversity and Evolutionary Research: from Genome to Ecosystem’ of MEXT; the Japan Society for the Promotion of Science (No. 18003246) to A.N.

References


(Received February 4, 2008; Accepted April 30, 2008)