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Kyoto University
BEACH-Domain Proteins Act together in a Cascade to Mediate Vacuolar Protein Trafficking and Disease Resistance in Arabidopsis

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Running title: BEACH-domain proteins of Arabidopsis thaliana

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ABSTRACT

Membrane trafficking to the protein storage vacuole (PSV) is a specialized process in seeds plants. However, this trafficking mechanism to PSV is poorly defined. Here, we show that three types of Beige and Chediak-Higashi (BEACH)-domain proteins contribute to both vacuolar protein transport and effector-triggered immunity (ETI). We screened a green-fluorescent seed (GFS) library of Arabidopsis mutants with defects in vesicle trafficking and isolated two allelic mutants gfs3 and gfs12 with a defect in seed protein transport to PSV. The gene responsible for the phenotype was a gene encoding a protein belonging to Group D of BEACH-domain proteins, which possess kinase domains. Disruption of other BEACH-encoding loci in the gfs12 mutant showed that BEACH homologs acted in a cascading manner for PSV trafficking. The epistatic genetic interactions observed among BEACH homologs were also reflected in the ETI responses of the gfs12 and gfs12 bchb-1 mutants, which showed elevated avirulent bacterial growth. The GFS12 kinase domain interacted specifically with the pleckstrin-homology domain of BchC1. These results suggest that a cascade of multiple BEACH-domain proteins contributes to vacuolar protein transport and ETI plant defence.

Keywords: BEACH domain protein; Arabidopsis thaliana; vacuolar protein transport; protein storage vacuoles; plant immunity.

INTRODUCTION

The cells of higher plants have two functionally distinct vacuoles (Paris et al., 1996; Neuhaus and Rogers, 1998), lytic vacuoles (LV, equivalent to lysosomes in mammals) which are found in all eukaryotic cells, and protein storage vacuoles (PSV), which are specific to higher plants and function as a storage site for storage proteins. The storage proteins constitute an essential source of amino acids for various biosynthetic activities during seed germination and also represent a major nutritional supplement for human and livestock (Okita and Rogers, 1996; North et al., 2010). Evidence for the coexistence of lytic and storage vacuoles in plant cells
came from the observation that antisera of γ-tonoplast intrinsic proteins (TIP) and α-TIP, which are markers for LV and PSV respectively, labelled vacuole membranes of two different compartments in the root tip squashes of barley and pea seedlings (Paris et al., 1996). However, the multiple-vacuole coexistence hypothesis was recently challenged by contradictory findings that fluorescently labelled γ-, α- and δ-TIP proteins localized to a common vacuolar location in Arabidopsis cells (Hunter et al., 2007; Frigerio et al., 2008).

Protein sorting to vacuoles with distinctive functions requires additional and specialised trafficking mechanisms. In mammalian and yeast cells, vacuolar sorting and delivery mechanisms share some fundamental features in that they are clathrin-dependent and involve interactions between the sorting receptor and AP1 adaptor complex (Ghosh et al., 2003; Nakayama and Wakatsuki, 2003). In mammalian cells, lysosomal acid hydrolases are sorted from secretory proteins at the trans-Golgi network (TGN) by the mannose-6-phosphate receptor (MPR) (Rohrer and Kornfeld, 2001). Mannose-6-phosphate receptor-ligand complexes are then packaged into clathrin-coated vesicles, a process facilitated by the monomeric GGAs (Golgi-localized, γ-ear containing ARF-binding proteins) and the tetrameric AP1 adaptor complex (Doray et al., 2002). Similarly, carboxypeptidase Y in yeast cells is transported to the vacuoles by Vps10p, a mammalian MPR analogue, through interaction between the cytoplasmic tail of Vps10p, an AP1 adaptor complex and GGAs (Costaguta et al., 2001; Deloche et al., 2001). We have shown that Arabidopsis AP-1 complex may facilitate the TGN-to-cell plate trafficking of a cytokinesis-specific syntaxin, KNOLLE, during cell division (Teh et al., 2013). This highlights the imminent needs to further delineate the already complicated vacuolar sorting mechanisms at the TGN.

In plant cells the coexistence of two functionally distinct vacuoles adds a layer of complexity to the vacuolar sorting mechanisms. Soluble proteins destined for the LV requires an NPIR type sequence-specific vacuolar sorting determinant (ssVSD) in order to be recognised by the vacuolar sorting receptor BP-80, although other non-NPIR type ssVSDs have also been documented (Kirsch et al., 1994; Saalbach et al., 1996; Matsuoka and Neuhaus, 1999; Frigerio et al., 2001). In contrast to ssVSDs found in LV-targeted proteins, no consensus motifs have been identified in the PSV-targeted proteins, probably due to the sequence heterogeneity in the storage proteins studied so far (Robinson et al., 2005). Sorting signals may not be the sole determining factor for proper PSV targeting. First, some storage
proteins such as ricin and castor bean 2S albumin are also found to harbor LV-specific ssVSDs and to interact with BP-80 in vitro (Frigerio et al., 2001; Jolliffe et al., 2004). Second, a vacuolar sorting receptor loss-of-function mutant in Arabidopsis, vsr1 (vacuolar sorting receptor1), missorted majority of the storage proteins to the extracellular space and a small portion of the storage proteins are still sorted normally (Shimada et al., 2003). Third, the vacuolar proteins in vsr1 were missorted non-specifically regardless of the type of sorting signals the cargoes carried (Craddock et al., 2008). Indirect evidence has suggested that trafficking pathways to LV and PSV could operate independently since trafficking to the LV was found to be selectively inhibited while PSV targeting remained unaffected (Bolte et al., 2004; Park et al., 2005; Sanmartin et al., 2007).

An effective approach to identify key components/gene products in vacuolar trafficking is systematic genetic screening, in which mutant lines that display compromised vacuolar trafficking (e.g. accumulation of premature/unprocessed forms of vacuolar proteins) from a mutagenized population are isolated and the responsible genes mapped. However, such an approach faces two hurdles. First, most vacuolar sorting mutants display no or pleiotropic macrophenotypes (Shimada et al., 2003; Yano et al., 2003; Niihama et al., 2009; Feraru et al., 2010; Pourcher et al., 2010; Zwiewka et al., 2011), which precludes the use of forward genetic screens by growth phenotypes. Second, functional redundancy between genes of the same family complicates the analysis of single loss-of-function mutants, making it difficult to assign a gene of interest to specific trafficking roles (Sanmartin et al., 2007; Zouhar et al., 2010). In order to identify novel components involved in plant vacuolar sorting mechanisms, we previously described a high-throughput screening system that used a PSV-targeted GFP reporter (Fuji et al., 2007). The GFP reporter is fused to CT24, a PSV targeting signal from the soybean β-conglycinin, and when expressed in Arabidopsis seeds, GFP-CT24 is transported to the PSV (Nishizawa et al., 2003; Fuji et al., 2007). GFP-CT24 has been validated to be an efficient reporter for vacuolar sorting deficiencies since expression in vacuolar sorting receptor1 (vsr1) caused GFP to be secreted and led to enhanced fluorescent in the vsr1 seeds (Fuji et al., 2007). The enhanced fluorescent phenotype in seeds with compromised vacuolar trafficking allows rapid isolation of putative mutant lines.

Here we characterise the green fluorescent seed12 (gfs12), which is a mutant line for the Beige and Chediak-Higashi (BEACH) domain gene. The BEACH domain is a ~280
amino acid-long domain that is highly conserved in the eukaryotes (Barbosa et al., 1996). BEACH-domain containing proteins are thought to act as scaffolds to facilitate membrane events such as vesicle fusion or fission in order to regulate trafficking, although direct evidence for its mechanistic actions remains elusive (Cullinane et al., 2013). In plants, the role of BEACH-domain protein in facilitating vacuolar proteins has not been demonstrated. Using genetics and biochemistry approaches we show that members of the BEACH family proteins act together in a cascade to mediate PSV-targeted trafficking and plant immunity in Arabidopsis.

RESULTS

GFS12 Encodes a BEACH-Domain Protein

The gfs12 mutant was isolated for its enhanced GFP fluorescence in the seeds when compared to the parental line GFP-CT24 (Figure 1A). The enhanced GFP fluorescence in gfs12 indicated that the PSV-targeted GFP was mis-sorted to intercellular space and implied that trafficking to the PSV is compromised. The GFS12 mutation is recessive and segregated in a simple Mendelian manner. gfs12 displayed no obvious growth defects throughout the life cycle. Unlike vsrl, which has previously been shown to abnormally accumulated the unprocessed precursor forms of two major PSV proteins 2S albumins and 12S globulins (Shimada et al., 2003; Fuji et al., 2007), gfs12 seeds accumulated predominantly the 12S globulins precursor (p12S) and trace amount of the 2S albumins precursor (p2S) when analysed on immunoblotting (Figure 1B). Since the accumulation of unprocessed 12S globulin is the predominant phenotype in gfs12, we decided to focus on this phenotype in the subsequent analysis.

Map-based cloning and whole genome sequencing of gfs12 identified a G-to-A transition at the 966th nucleotide in locus At5g18525, which encodes a protein with a Beige and Chediak-Higashi (BEACH) domain (Figure 2A). The BEACH domain is named after a rare autosomal genetic disorder called Chediak-Higashi syndrome (Barbosa et al., 1996) and defines a large family of genes that are conserved across all eukaryotes. The BEACH domain proteins are usually very large in size and share a similar structural organisation (Figure 2A).
Though the function of the BEACH domain is unknown, BEACH domain proteins have been implicated in diverse cellular processes such as vesicle trafficking, cytokinesis and receptor signalling (Kwak et al., 1999; Su et al., 2004; Khodosh et al., 2006). For the ease of discussion, we subsequently renamed the At5g18525 locus as *GFS12*. *GFS12* encodes a 1637 amino acid polypeptide with two putative kinase domains flanking the 232-amino acid long BEACH domain. Like all other BEACH proteins, the extreme C-terminus of GFS12 harbors the highly conserved WD40 repeats which have regulatory roles and are involved in protein-protein interactions (Neer et al., 1994). The EMS-induced G-to-A transition in *GFS12* replaced the 322th tryptophan codon with a stop codon and created a premature termination before the BEACH domain (Figure 2A). We subsequently identified an additional *GFS12* allele from the same genetics screen, *gfs3* which has a Trp-1239-stop nonsense point mutation in *GFS12* (Figure 2A). *gfs3* was therefore renamed as *gfs12-2*. *gfs12-2* shared the p12S accumulation phenotype as *gfs12* (Figures 1B). Both *gfs12* and *gfs12-2* were null alleles as we were unable to detect *GFS12* transcripts in the RT-PCR analyses (Figure 2B). To confirm that the point mutations in the *gfs12* mutants were indeed responsible for the p12S accumulation phenotype, we transformed *gfs12* and *gfs12-2* plants with a 35S promoter-driven GFS12-RFP (red fluorescent protein) fusion protein and showed that the phenotype was complemented by an exogenous wild-type copy of *GFS12* (Figure 2C and Supplemental Figure 1). This provides direct evidence that the disruption of *GFS12* caused the p12S accumulation in *gfs12* mutants and that the GFS12-RFP fusion protein is functional.

The p12S accumulation in *gfs12* seems to be a highly specific trafficking defect that is found in the seeds but not in vacuolated vegetative tissues. Stable expression of the secretory marker secRFP (Zheng et al., 2004) and the tonoplast marker GFP-γ-TIP (Hunter et al., 2007) in *gfs12* showed that these marker proteins were correctly trafficked to the intercellular space and vacuolar membrane, respectively (Supplemental Figure 2A-2D). Therefore, defects in the membrane trafficking of these fluorescent markers in vegetative cells were not detected.

We next investigated the tissue expression pattern of *GFS12* using a promoter-GUS fusion gene (*GFS12*pro::GUS). In *Arabidopsis* plants expressing *GFS12*pro::GUS, GUS activities were weakly found in the cotyledons of germinating seedlings (Supplemental Figure 3A). GUS-staining showed that *GFS12* expression was restricted to the vasculature tissues of cotyledons and root tissues. *GFS12* was also expressed at the apical meristem, root tip, young
flower buds, and receptacles (Supplemental Figure 3).

The *Arabidopsis* BEACH-Domain Proteins Can Be Classified into Four Groups

The *Arabidopsis* genome contains six BEACH domain-containing genes, namely *GFS12* (At5g18525), *SPIRRIG* (At1g03060), At4g02660, At1g58230, At2g45540 and At3g60920 (Saedler et al., 2009). Only one of these genes, *SPIRRIG* (At1g03060), has been characterised so far. The *SPIRRIG* gene has a role in cell morphogenesis as the *sprrig* (*spi*) mutant displayed pleiotropic cellular distortion. Vacuoles in *spi* root hairs, like PSV in *gfs12* seeds, are highly fragmented (Saedler et al., 2009). In a phylogenetic tree of BEACH-domain proteins (Supplemental Table 1), *Arabidopsis* BEACH proteins fell into four groups, A, B, C and D (Figure 3). At3g60920 and At2g45540 were in Group C, while At4g02660 and SPIRRIG/At1g03060 were in Group A. The tree shows that the GFS12/BchD diverged from other mammalian and yeast BEACH proteins to form an outgroup that is characterised by plant-specific BEACH proteins with kinase domains (Supplemental Figure 4), suggesting this group of BEACH protein has evolved to acquire plant-specific functions (Figure 3, Group D). Moreover the bootstrap value of 70 for the Group D provides additional evidence for the outbranching of the group (Figure 3). Interestingly, GFS12 is the only *Arabidopsis* BEACH protein that possesses kinase domains that flank the BEACH domain (Figure 4A). A phylogenetic analysis based on the PH-BEACH-WD40 domains arrived at a similar conclusion (Saedler et al., 2009), although At3g60920 was not included in the analysis because it lacks the WD40 repeats.

Genetic Interactions between *GFS12* and BEACH Homologs

We next investigated the possible involvement of BEACH protein homologs in PSV sorting. To this end, we isolated T-DNA knock-out or knock-down mutants (Supplemental Figure 5) from every BEACH domain-encoding gene in *Arabidopsis* and analysed the seed protein profiles. In summary, while *gfs12* accumulated p12S (Figure 4B), none of the mutant alleles of *AtBchA1*, *AtbchA2*, *AtBchC1* and *AtBchC2* accumulated either p12S or p2S. One allele from the *AtBchB*, *bchb-1* weakly accumulated p12S only (Figure 4B).
We further examined the possible genetic interactions between \textit{GFS12/BchD} and other BEACH protein homologs. We therefore selected one representative BEACH member from Groups B and C to generate double mutants \textit{gfs12 bchb-1} and \textit{gfs12 bchc1-1}. We found that p12S accumulation by the double mutant \textit{gfs12 bchb-1} was greatly enhanced and similar to that in \textit{vsr1-2} (Figure 5A). Accordingly p2S also began to highly accumulate in \textit{gfs12 bchb-1}, a phenotype which was not observed in the \textit{gfs12}, indicating that \textit{AtBchB} functionally overlaps with GFS12/BchD to mediate 12S globulins and 2S albumins trafficking to the PSV (Figure 5A). Surprisingly, knocking out \textit{AtBchC1} in \textit{gfs12} rescued the p12S accumulation, as the \textit{gfs12 bchc1-1} double mutant no longer accumulated the p12S (Figure 5A). The genetic suppression observed in the \textit{gfs12 bchc1-1} double mutant indicates that \textit{AtBchC1} plays an epistatic role to GFS12. The absence of p12S in seeds of the \textit{bchc1-1} single mutant (Figure 4B) suggests that \textit{BchC1} acts downstream of \textit{GFS12} at the PSV-targeted trafficking. Therefore the most likely explanation for the suppression phenotype in \textit{gfs12 bchc1-1} would be that GFS12/BchD acts to inhibit BchC1, which is a negative regulator of PSV-targeted trafficking (see Figure 8).

The overlapping/redundant function between GFS12/BchD and BchB prompted us to ask the question whether BchB acts independently or through inhibiting the BchC1 to mediate PSV trafficking. To distinguish these two possibilities genetically, we examined the level of p12S and p2S accumulation in the seeds of triple mutant \textit{gfs12 bchb-1 bchc1-1}. The enhanced p12S and p2S accumulations seen in the double mutant \textit{gfs12 bchb-1} were greatly or totally eliminated, respectively by the \textit{bchc1-1} mutation (Figure 5A), indicating that BchB functions in parallel with the GFS12/BchD to inhibit the BchC1 (see Figure 8). Surprisingly, the BEACH homologs do not seem to be essential for the general plant growth as mutants of all combinatorial mutations displayed normal development throughout their life cycle (Figure 5B).

\textbf{The GFS12/BchD Kinase Domain Physically Interacts with specific sites of the Pleckstrin Homology Domain of BchC1}

The BchB and BchC1 proteins each possesses a pleckstrin homology (PH) domain, which is known to physically interact with a wide range of signalling molecules such as
phosphoinositols and kinases to facilitate membrane targeting and trafficking (Yao et al., 1994; Lemmon, 2004). Since GFS12/BchD is characterised by two kinase domains, we therefore ask the question does the kinase and BEACH domains in GFS12/BchD interact with BchB and/or BchC1 PH domains. Using the yeast two hybrid assay, we examined the interaction between N-terminal kinase domain I (K1), C-terminal kinase domain II (K2) and BEACH domain (Bch) of GFS12/BchD with PH domains of BchB (B PH-Bch) and BchC1 (C1 PH-Bch) (Figure 4A). All yeast strains harboring the bait/prey empty vectors did not show auto-activation when transformed with the designated bait/prey proteins (Figure 6A, top most and left most panels). The GFS12/BchD K1, K2 and Bch proteins have no detectable interaction amongst themselves (Figure 6A). We further tested all the possible combinations of bait and prey proteins between GFS12/BchD, BchB and BchC1 and only interaction between K2 and C1 PH-Bch was detected (Figure 6A). We confirmed this interaction by swapping the prey and bait cloning vector between K2 and C1 PH-Bch (Supplemental Figure 6).

Previously the crystal structure of a human BEACH homolog Neurobeachin (Nbea) has been determined at the 2.9 Å resolution (Jogl et al., 2002). The Nbea PH domain is able to interact with its own BEACH domain via an extensive interface. This interaction is highly dependent on the conserved amino acid residues distributed in the PH and BEACH domains (Jogl et al., 2002). To clarify a specific interaction between the GFS12/BchD K2 and BchC1 PH-Bch domains, we introduced mutations at two highly conserved amino acids each in the PH and BEACH domains of BchC1. When R1615E (corresponds to R2208 in Nbea) and E1625R (corresponds to E2218 in Nbea) mutations were introduced in the BchC1 PH domain, the interaction between GFS12/BchD K2 and C1 PH-Bch was abolished (Figure 6B). On the contrary, the interaction remained unchanged in the N1704A (corresponds to N2302 in Nbea) and Q1708A (corresponds Q2306 in Nbea) BEACH mutants (Figure 6B). We therefore concluded that the R1615 and E1625 amino acids in the BchC1 PH domain are crucial for the physical interaction with K2 domain of GFS12/BchD.

GFS12/BchD and BEACH Proteins Are Required for Effector-Triggered Immunity

Endosomal trafficking components have recently been implicated in the plant immunity
response upon pathogenesis (Beck et al., 2012; Uemura et al., 2012). Therefore we investigated the possible involvement of BEACH proteins in mediating effector-triggered immunity (ETI) induced by *Pseudomonas syringae* pv. *tomato* (*PstDC3000/avrRpm1*). In control CT24 parental plants, growth of the avirulent bacteria was $10^4$ cfu/ml, whereas it was two orders of magnitude higher in *gfs12* ($10^6$ cfu/ml, Figure 7A). The compromised immune response in *gfs12* was GFS12-dependent, as a proper ETI response was restored in the complemented *gfs12* lines (Figure 7B). Analysis of the ETI response in double mutants of BEACH homologues revealed an interesting correlation with the PSV trafficking defects described earlier. In *gfs12 bchc1-1*, in which p12S accumulation was suppressed, the compromised ETI response was also inhibited and exhibited the same bacterial titre as the control parental plants (Figure 7A). On the other hand, *gfs12 bchb-1* showed a severed ETI response (bacterial growth titre $10^6$ cfu/ml. Figure 7A), which correlates well with the enhanced p12S and p2S accumulation phenotype.

To further delineate the involvement of BEACH domain proteins in ETI, we examined the ETI-associated hypersensitive cell death in *gfs12* by ion leakage assay upon inoculation with *PstDC3000/avrRpm1*. The ion leakage assay in which *gfs12, gfs12 bchc1-1, gfs12 bchb-1* and the complemented *gfs12* showed elevated conductance, similar to that in the control parental plants, indicating that all the BEACH mutants were able to initiate hypersensitive cell death (Figure 7C). We therefore concluded that the BEACH proteins play an essential role in ETI but not in the cell death.

**DISCUSSION**

Since the identification of the first BEACH-domain containing protein in 1996, the protein family represents an enigmatic class of trafficking-related protein with elusive functions (Barbosa et al., 1996). Extensive characterization of the mammalian BEACH homolog Lysosomal Trafficking Regulator (LYST) has revealed a role in the neuronal cell lysosomal trafficking, however the mechanisms and functional role of the BEACH domain in mediating lysosomal trafficking are still poorly defined (Wang et al., 2000; de Souza et al., 2007; Lim and Kraut, 2009). BEACH proteins have a large size (>400 kDa) which makes
characterization difficult and therefore the subcellular localization of the BEACH homologs has been elusive. Although BEACH proteins do not have transmembrane domains, subcellular fractionation of the yeast BEACH homolog, Beige Protein Homologue 1, suggests that it is a membrane-associated protein (Shiflett et al., 2004). In an immunolocalization experiment, the mammalian BEACH homolog Neurobeachin was shown to label unidentified polymorphic tubulovesicular endomembranes (Wang et al., 2000). The *Dictyostelium* and *Drosophila* BEACH homologs are lysosome-associated, which indicates that they have roles in lysosomal trafficking (Kypri et al., 2007; Lim and Kraut, 2009). Whether these differential localizations between the BEACH homologs represent their functional divergence or a novel cellular compartment specific to the BEACH protein remain to be determined.

The epistatic genetic interactions observed between GFS12/BchD, BchB and BchC1 highlight the hierarchical/cascading actions of the BEACH homologs in negatively mediating PSV trafficking and plant ETI (Figure 8). We propose that GFS12/BchD acts predominantly to suppress BchC1, which itself is a negative factor in PSV trafficking regulation and plant immunity response (Figure 8). Furthermore, the additive effects of *bchb-1* mutation on the PSV trafficking phenotype in *gfs12-1* (Figure 5) is a strong indication that GFS12 and BchB act to suppress BchC1. Multiple independent trafficking pathways to vacuoles have recently been described (Ebine et al., 2014; Singh et al., 2014). Although our genetic analysis and our yeast two hybrid analysis (see below) support the proposed working model, we cannot rule out the possibility that BchC1 might act as an negative regulator of an entirely independent PSV trafficking pathway where elimination of BchC1 results in bypassing the GFS12- and BchB-dependent pathway.

Our yeast two hybrid analysis (Figure 6) shows that Kinase II domain (K2) of GFS12/BchD specifically interacts with the BchC1 PH domain. Based on this interaction, we propose a working model of the inhibitory effects of BEACH proteins on PSV trafficking and plant immunity response (Figure 8). In this working model, the GFS12/BchD Kinase II domain phosphorylates BchC1 via protein-protein interaction mediated by the PH domain in BchC1. Phosphorylation of BchC1 by the GFS12/BchD will induce conformational changes in BchC1 that will inactivate it. Further studies using X-ray crystallography on the phosphorylated of BchC1 upon phosphorylation is necessary to prove the case. Our working model also implies that the expression level of BchC1, a negative regulator, should be kept at
the lowest to ensure PSV trafficking and immunity response is minimally inhibited. In consistent with this, the mean expression levels of BchC1 is the lowest compared to that of GFS12/BchD and BchB (Supplemental Figure 7) (Obayashi et al., 2011). The relatively higher expression levels of the GFS12/BchD and BchB compared to the BchC1 would ensure that the inhibitory effects of BchC1 are kept in check.

Interestingly, all three BEACH proteins characterised in this study exert inhibitory effects in a cascade to fine tune PSV trafficking as shown in Figure 8. Notably, BEACH homologs from other model organisms seem to have similar regulatory effects. For example, the Dictyostelium LvsB and Drosophila MAUVE (a LYST homolog) act as negative regulators to limit the homotypic and heterotypic fusions of early endosomes with postlysosomal compartments (Kypri et al., 2007; Rahman et al., 2012); a C. elegans BEACH homolog, SEL-2 (a neurobeachin homolog), negatively regulates the Notch activity in polarised epithelial cells (de Souza et al., 2007) and finally the Drosophila Blue Cheese antagonises the small GTPase Rab11 during synapse morphogenesis (Khodosh et al., 2006). These findings exemplified a common feature of the BEACH proteins: they are the negative regulators of lysosomal trafficking.

All BEACH mutants reported so far share a diagnostic phenotype which is an enlarged lysosome (Introne et al., 1999). This mutant phenotype has led to the suggestion that BEACH proteins function to limit the homotypic fusions of the lysosomal compartments. The lysosome equivalent in plants is the lytic vacuole. Using the vacuolar membrane marker GFP-γ-TIP, we confirmed that the morphology of the lytic vacuole remained unchanged in the gfs12 mutant (Supplemental Figure 2C and 2D).

Interestingly, the compromised ETI phenotypes of gfs12 bchc1-1 and gfs12 bchb-1 correlate well with the PSV trafficking defects in these mutants. This observation is unexpected, since the gfs12 and bchb-1 mutations only impacted on storage protein trafficking in seeds while no other discernible trafficking defects was observed in mature rosette leaves where ETI takes place. A possible explanation for this unexpected observation would be that the ubiquitously-expressed BEACH-related proteins GFS12 and BchB (Supplemental Figure 7) function at PSV trafficking in embryos and defence-associated trafficking in rosette leaves. In support of this, mounting evidence showed that membrane trafficking proteins play substantial roles in plant immunity (Teh and Hofius, 2014). It is also interesting to note that
although \textit{gfs12 bchb-1} showed compromised ETI, \textit{avrRpm1}-conditioned HR-cell death was not affected, implying that GFS12 and BchB act specifically in ETI which could be uncoupled with the HR cell death (Heidrich et al., 2011; Teh and Hofius, 2014).

In conclusion, the data presented here demonstrated that \textit{Arabidopsis} BEACH proteins are multifunctional proteins that are required for PSV trafficking and plant immunity. By acting in a cascade manner, the BEACH proteins form a hierarchy network that helps to fine tune PSV trafficking during seed development. Further delineation of the protein functions would require substantial knowledge on the subcellular localization of the BEACH proteins as well as careful dissection of the conserved BEACH domains.

\section*{METHODS}
\subsection*{Plant Materials and Growth Conditions}
\textit{Arabidopsis} T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center. A list detailing all lines used in this study is described in the supplemental information.

\subsection*{Mapping and Next Generation Sequencing}
The \textit{gfs12} mutations were roughly mapped to a 200-kb region on chromosome 5, and the genomic DNA was analyzed by next generation sequencing as described in (Tabata et al., 2012) to identify the mutations.

\subsection*{Arabidopsis Complementation}
A genomic full-length fragment of \textit{GFS12} was amplified using primers GFS12-infusion-F (5’ aaccaatctcagtcgacatgagaggtgaagatagtgacctttgc3’) and GFS12-infusion-CR (5’aagctgggtctagatatccacaacagattctcaagtgaccatcatg3’). The fragment was cloned into entry vector pENTR1A (Invitrogen) using an In-Fusion Cloning Kit (Clontech). To generate 35S\textsubscript{pro}:GFS12-RFP, the GFS12 genomic fragment was subcloned to pGWB560 by Gateway\textsuperscript{®} LR reactions. A complementation experiment was carried out by transforming the \textit{gfs12} plants with 35S\textsubscript{pro}:GFS12-RFP using the floral dip method; primary transformants were selected on hygromycin-containing MS media and propagated to T3 generations in order to obtain
homozygous lines. To generate a GUS reporter line for the GFS12 promoter, a 2-kb genomic fragment upstream of the GFS12 start codon was amplified using primers pGFS12-infusion-F (5’aaccaatcagtcgacacgcagatatgagcgacggaatcc3’) and pGFS12-infusion-R (5’aagctgggtctagatatccccctttctttgaggaagagag3’). The PCR fragment was cloned into pENTR1A by an In-Fusion cloning kit and further subcloned to pHGWFS7 to generate GFS12pro:EGFP-GUS.

**GUS Staining and Drug Treatment**

Seedlings and tissues of transgenic lines harboring GFS12pro:EGFP-GUS were incubated on ice in cold 90% acetone for 15 min, vacuum infiltrated for 15 min in the GUS staining solution (0.5 mg/ml X-Gluc, 100 mM NaH2PO4, 10 mM EDTA, 0.1% Triton X-100, 5 mM K4[Fe(CN)6]) to improve its penetration into tissues, incubated overnight at 37°C in the staining solution, transferred to 70% ethanol and destained in ethanol: acetic acid (6:1) with gentle shaking. Four to 5-day old seedlings were used for confocal imaging experiments. For FM4-64 (an endocytic tracer that labels endosomes) staining, seedlings were incubated in 5 µM FM4-64 for 5 min at room temperature and washed briefly in sterile water before proceeding to imaging. To visualise BFA compartments (agglomerations of endosomes and trans-Golgi network due to BFA treatment), seedlings were pre-stained with FM4-64 before incubating in 50 µM BFA solution for 1 h.

**SDS-PAGE and Immunoblots**

Seed proteins were extracted from a pool of 50 seeds in 50 µl of extraction buffer [50 mM Tris-HCl (pH6.8), 250 mM NaCl, 25 mM EDTA, 50% v/v glycerol, 0.5% v/v 2-mercaptoethanol, 1% v/v SDS]. Proteins from 5-8 seeds was analysed on SDS-PAGE. 12S globulin and 2S albumin were immunodetected with rabbit anti-12S and anti-2S antibodies at 10,000 x and 5,000 x dilutions.

**Pathogen Strains, Pathogen Tests and Evaluation of Cell Death**

The avirulent bacterial strain *Pseudomonas syringae* pv. tomato DC3000/avrRpm1 (*PstDC3000/avrRpm1*) was used in this study. Leaves were infiltrated with freshly cultured *PstDC3000/avrRpm1* as described previously (Hatsugai et al., 2009). Plant immunity and cell death caused by *PstDC3000/avrRpm1* were assayed by measuring the bacterial growth and
ion leakage, respectively (Hatsugai et al., 2009).

**Yeast-Two-Hybrid Assay**

Yeast strain AH109, expression cassettes pDEST-GADT7 (Rossignol et al., 2007) and pDEST-GBKT7 (Rossignol et al., 2007) were used throughout this study. cDNA of specific domains from GFS12, BchB and BchC1 were amplified by PCR (Supplemental Table 2), sequenced and cloned into the expression cassettes by Gateway cloning. To co-transform the yeast strain AH109, a 100 mL culture (subcultured from an overnight culture) was grown to OD<sub>600</sub> 0.5, centrifuged at 1500 x g for 5 min, washed in distilled water and resuspended in 1 mL 0.1 M Lithium acetate/TE buffer. Twenty-five µL of yeast suspension cells were aliquoted for each transformation. Five µL sonicated salmon sperm carrier DNA (2.5 mg/mL), 1 µg of each plasmid DNA and 200 µL PEG-4000 (50% w/v) were added to the cells in that order. The mixture was vortexed vigorously and incubated at room temperature for 1 h. The cells were by heat shock at 42°C for 1 h, washed once in 800 µL distilled water, plated in SD-W-L media and grown for 3 days to select for yeast strains that harbor both expression cassettes. To test for protein-protein interaction, yeast strains co-expressing bait and prey proteins were plated on SD-W-L-H-A media for 4 days.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Abnormal accumulation of 12S globulin precursor (p12S) in gfs12-2/gfs3 is complemented by a RFP-fusion protein of GFS12.

**Supplemental Figure 2.** Secretory and Lytic Vacuole Trafficking Pathways Were Unaffected in gfs12.

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**FUNDING**

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**ACKNOWLEDGEMENTS**

We thank Dr. Sota Fujii (Kyoto University) for advice on phylogeny analysis.
Figure Legends

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Supplemental information

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Supplemental Table 2. DNA sequence of primers used in the semi quantitative RT-PCR of BEACH-domain T-DNA mutants and yeast-two-hybrid assay.

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