Overexpression of BAX INHIBITOR-1 Links Plasma Membrane Microdomain Proteins to Stress

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BAX INHIBITOR-1 (BI-1) is a cell death suppressor widely conserved in plants and animals. Overexpression of BI-1 enhances tolerance to stress-induced cell death in plant cells, although the molecular mechanism behind this enhancement is unclear. We recently found that Arabidopsis (Arabidopsis thaliana) BI-1 is involved in the metabolism of sphingolipids, such as the synthesis of 2-hydroxy fatty acids, suggesting the involvement of sphingolipids in the cell death regulatory mechanism downstream of BI-1. Here, we show that BI-1 affects cell death-associated components localized in sphingolipid-enriched microdomains of the plasma membrane in rice (Oryza sativa) cells. The amount of 2-hydroxy fatty acid-containing glucosylceramide increased in the detergent-resistant membrane (DRM; a biochemical counterpart of plasma membrane microdomains) fraction obtained from BI-1-overexpressing rice cells. Comparative proteomics analysis showed quantitative changes of DRM proteins in BI-1-overexpressing cells. In particular, the protein abundance of FLOTILLIN HOMOLOG (FLOT) and HYPERSENSITIVE-INDUCED REACTION PROTEIN3 (HIR3) markedly decreased in DRM of BI-1-overexpressing cells. Loss-of-function analysis demonstrated that FLOT and HIR3 are required for cell death by oxidative stress and salicylic acid, suggesting that the decreased levels of these proteins directly contribute to the stress-tolerant phenotypes in BI-1-overexpressing rice cells. These findings provide a novel biological implication of plant membrane microdomains in stress-induced cell death, which is negatively modulated by BI-1 overexpression via decreasing the abundance of a set of key proteins involved in cell death.

BAX INHIBITOR-1 (BI-1) is an endoplasmic reticulum (ER)-based cell death suppressor widely conserved in plants and animals (Xu and Reed, 1998; Kawai et al., 1999). In plants, BI-1 is considered a stress-associated factor, since its expression is stimulated by various stresses (Sanchez et al., 2000; Kawai-Yamada et al., 2001; Matsumura et al., 2003; Watanabe and Lam, 2006; Isbat et al., 2009). Although plants lack the homolog of animal BAX as an inducer of programmed cell death, loss of BI-1 expression results in a severe cell death phenotype under stress conditions, such as fumonisin B1-induced ER stress and disturbance of ion homeostasis (Watanabe and Lam, 2006; Ihara-Ohori et al., 2007). Conversely, plants overexpressing BI-1 exhibit tolerance to cell death induced by various stresses (Kawai-Yamada et al., 2001, 2004; Matsumura et al., 2003; Ihara-Ohori et al., 2007; Watanabe and Lam, 2008; Ishikawa et al., 2010). Moreover, BI-1 overexpression confers not only tolerance to oxidative stress-mediated cell death but also enhanced metabolic acclimation involved in energy and redox balance (Ishikawa et al., 2010). The results of these studies indicate that plant BI-1 is potentially useful for engineering stress-tolerant plants. However, little is known about the mode of action of BI-1 in the cell death regulatory pathway (Ishikawa et al., 2011). While overexpression systems sometimes include artificial or off-site effects, the observation that BI-1 overexpression improves stress tolerance suggests the importance of dissecting plants overexpressing it to further address the molecular basis of BI-1 function and cell death and stress tolerance management.

As another approach to understand the molecular function of BI-1, screening of candidates interacting biochemically or functionally with BI-1 has been performed. First, Arabidopsis (Arabidopsis thaliana) BI-1 was confirmed to bind to calmodulin, like barley (Hordeum vulgare) MLO protein, a membrane-bound cell death regulator (Kim et al., 2002; Ihara-Ohori et al., 2007). Since the calmodulin-binding ability of BI-1 and MLO is necessary for their cell death-suppressing activity, Ca²⁺ signaling is critically involved in BI-1- and MLO-mediated cell death regulation (Kim et al., 2002; [OPEN]1Articles can be viewed without a subscription. www.plantphysiol.org/cgi/doi/10.1104/pp.15.00445
More recently, it was also demonstrated that the cell death suppression by BI-1 is mediated, at least in part, through fatty acid hydroxylase (FAH) in a *Saccharomyces cerevisiae* ectopic expression system (Nagano et al., 2009). In addition, Arabidopsis FAHs (AtFAH1 and AtFAH2) interact with BI-1 via cytochrome *b*₅ at the ER, resulting in the accumulation of 2-hydroxy fatty acids (2-HFAs) in Arabidopsis plants overexpressing BI-1. 2-HFAs are typical components of the ceramide backbone of sphingolipids (Imai et al., 1995; Pata et al., 2010). Although many functions of plant sphingolipids remain to be elucidated, accumulating evidence clearly indicates that sphingolipids and their metabolism are closely involved in cell death regulation and various stress responses in plants (Ng et al., 2001; Liang et al., 2003; Townley et al., 2005; Chen et al., 2008; Wang et al., 2008; Saucedo-García et al., 2011; Dutilleul et al., 2012; König et al., 2012; Nagano et al., 2012; Mortimer et al., 2013), implying that BI-1 plays a role in cell death regulation through sphingolipid metabolism. Sphingolipids are major components of membrane lipids and are at particularly high concentrations in membrane microdomains, known as lipid rafts in animal cells, which are essential for membrane-mediated signaling and act as a sorting platform for targeted protein traffic (Simons and Toomre, 2000; Staubach and Hanisch, 2011). In mammalian cells, sphingomyelin metabolism in lipid rafts plays a vital role in the initiation of apoptotic cell death (Milhas et al., 2010). Recent studies have demonstrated the presence of raft-like membrane microdomains in plant cells and a role for them in defense responses and targeted protein sorting (Peskan et al., 2000; Fujiwara et al., 2009; Minami et al., 2009; Melser et al., 2010; Markham et al., 2011).

This study focused on membrane microdomains in relation to BI-1-mediated sphingolipid metabolism. Our findings indicated that BI-1 alters sphingolipid composition in membrane microdomains, and this is accompanied by dynamic changes in a number of detergent-resistant membrane (DRM) proteins involved in cell death regulation.

**RESULTS**

**BI-1 Overexpression in Rice Cells Leads to the Accumulation of 2-HFAs as the Acyl Moiety of Sphingolipids**

To investigate the effects of BI-1 on lipid metabolism and membrane microdomain components, we used a rice suspension culture line in which the overexpression of Arabidopsis BI-1 significantly enhances the tolerance to cell death induced by elicitors, salicylic acid (SA), and oxidative stress (Matsumura et al., 2003; Ishikawa et al., 2010). The suspension culture system allows harvesting the large number of homogenous samples required for the biochemical preparation of membrane microdomain components. We first analyzed changes in the 2-HFA composition of glucosylceramide (GlcCer), one of the major plant sphingolipid classes. Gas chromatography-mass spectrometry (GC-MS) analysis of fatty acyl moieties of purified GlcCer fractions showed that rice GlcCer contains three major 2-HFA species with saturated acyl chains of 20 to 24 carbons (i.e. 20h:0, 22h:0, and 24h:0). These three species made up around 20% of the total fatty acid of GlcCer. Other minor 2-HFA species and nonhydroxy fatty acids made up only 10% and 5%, respectively (Supplemental Table S1). Of the major fatty acid species, the 20h:0 content in BI-1-overexpressing (OX) cells was significantly elevated, up to 18% to 33% higher than in wild-type cells, whereas 22h:0 and 24h:0 did not change (Fig. 1, A and B; Supplemental Table S1). Since 20h:0 is the predominant species of rice GlcCer (approximately 50%), its accumulation resulted in an increase in the total amount of 2-HFA-containing GlcCer (Fig. 1C). This finding indicated that BI-1 overexpression enhances the accumulation of 2-HFA as a constituent of

**Figure 1.** GC-MS analysis of fatty acyl moieties of GlcCer fractions from rice cells. Fatty acid methyl esters liberated from GlcCer fractions by methanolysis were analyzed by GC-MS. A, Content of GlcCer fatty acid species. Other HFA includes minor species; NFA shows the total content of nonhydroxy fatty acids. For quantitative data for all species, see Supplemental Table S1. B, Relative abundance (OX/wild type [WT]) of the three major fatty acid species. C, Total fatty acid content in GlcCer fractions. Data are means ± se (*n* = 4). FW, Fresh weight.
sphingolipids in rice suspension cells, as it does in Arabidopsis plants (Nagano et al., 2009).

Preparation and Analysis of DRM from BI-1 OX Rice Cells

Next, we addressed whether the accumulation of GlcCer in BI-1 OX cells affects membrane structures, particularly components of membrane microdomains, in which sphingolipid serves as a basal structural lipid. As a starting material for microdomain preparation, the plasma membrane (PM) was isolated from suspension cells of the wild type and BI-1 OX line 2, which showed the largest increase in total GlcCer, as shown in Figure 1. Enzyme assays (Supplemental Table S2) and western-blot analysis (Supplemental Fig. S1A) of marker proteins validated the purity of the PM fractions from both wild-type and OX cells. Microdomains of the PM fractions were prepared using a conventional method based on detergent insolubility (i.e. cold Triton X-100 treatment followed by Suc gradient centrifugation), which yielded the PM-derived DRM fraction. The recovery of the DRM from PM on a protein content basis was comparable between wild-type and OX cells (Supplemental Fig. S1B). Thin-layer chromatography analysis demonstrated a characteristic lipid composition of the DRM fractions prepared from both wild-type and OX cells (Table I): rich in GlcCer and sterols but poor in phospholipids and other neutral lipids, compared with the PM. This is fairly consistent with the well-characterized DRM lipid composition of various plants as well as other organisms, although the enrichment of GlcCer in the DRM fraction seems lower in rice than that reported in Arabidopsis and tobacco (Nicotiana tabacum; Mongrand et al., 2004; Borner et al., 2005; Minami et al., 2009). However, the GlcCer content was slightly, but statistically, significantly (P < 0.05) higher in the DRM fractions prepared from BI-1 OX cells compared with the wild type, whereas there was no significant difference in the PM-GlcCer content between the two cell lines. GC-MS analysis of the fatty acid composition of GlcCer in the PM and DRM fractions showed that 20h:0-containing GlcCer was highly accumulated in DRM fractions in BI-1 OX cells but not in wild-type cells, whereas GlcCer species with longer fatty acyl chains (22h:0 and 24h:0) were similarly enriched in the DRM fractions of wild-type and OX cells (Supplemental Fig. S1C). The composition of other major lipids in both the PM and DRM fractions was comparable between wild-type and OX cells. Taken together with lipid analyses of total lipid extracts and membrane fractions, these findings suggest that the sphingolipids with altered composition in BI-1 OX cells are enriched in PM microdomains.

Table I. Lipid composition of PM and PM-derived DRM fractions

Data are means ± so (n = 6–7). Other neutral lipids are mainly diacylglycerol, triacylglycerol, and free fatty acids.

| Lipid          | Wild Type | OX    | DRM | Wild Type | OX    | DRM:PM Ratio | Wild Type | OX    | mol %       |
|----------------|-----------|-------|-----|-----------|-------|--------------|-----------|-------|-------------|
| GlcCer         | 11.1 ± 2.0| 11.3 ± 1.3| 13.0 ± 1.0| 15.6 ± 1.0 | 1.17 ± 0.09 | 1.38 ± 0.09 |
| Sterylglucosides| 9.4 ± 1.1 | 8.8 ± 2.0 | 12.0 ± 1.3| 11.6 ± 2.1 | 1.28 ± 0.07 | 1.32 ± 0.12 |
| Acylated sterylglucosides | 11.5 ± 1.9 | 10.5 ± 1.4 | 21.0 ± 3.2| 19.5 ± 2.2 | 1.82 ± 0.14 | 1.87 ± 0.11 |
| Free sterols   | 16.3 ± 2.7| 15.8 ± 0.9 | 24.8 ± 1.9| 23.4 ± 2.3 | 1.52 ± 0.06 | 1.48 ± 0.07 |
| Phospholipids  | 43.7 ± 2.7| 46.2 ± 2.5 | 24.0 ± 2.4| 24.7 ± 1.6 | 0.55 ± 0.03 | 0.54 ± 0.02 |
| Other neutral lipids | 8.0 ± 3.1 | 7.6 ± 2.5 | 5.2 ± 1.3 | 5.2 ± 1.2 | 0.65 ± 0.08 | 0.68 ± 0.08 |

P < 0.05, compared with the value of the respective wild-type cells.

Different Protein Profiles in DRM Fractions of BI-1 Overexressor

The main focus of this study was to determine the effects of altered sphingolipid metabolism on components and functions of PM microdomains in BI-1 OX cells. Comparison of the protein profiles of the DRM fractions by SDS-PAGE showed different intensities of three major bands (A, B, and C) in wild-type and OX cells (Fig. 2). Moreover, the altered proteins were enriched in the DRM compared with PM. Similar DRM-specific protein profiles were reproducibly obtained in independent preparations from the two BI-1 OX lines that showed higher 2-HFA contents as described above (Supplemental Fig. S1D). In contrast to the marked changes in DRM proteins, no significant differences were found in SDS-PAGE profiles of microsomal membrane proteins, either Triton-soluble or -insoluble fractions (Supplemental Fig. S1E). These results also indicated that BI-1 predominantly affects the PM microdomain components, and not only lipids but also proteins.

To identify the DRM proteins that showed different intensities in SDS-PAGE bands, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in two ways: one focusing on the visibly different protein bands, and the other a shotgun analysis targeting whole proteins of the DRM fractions. First, the three bands with different intensities between wild-type and BI-1 OX cells (Fig. 2) were excised from gels and analyzed by LC-MS/MS. This approach allowed the identification of several proteins in each band (Supplemental Table S3). Taking into account the number and mass spectrometry (MS) intensities of the assigned peptides, four proteins were determined to be...
the predominant ones in the three bands. From bands A and C, which decreased in intensity, two distinct members of band 7 family proteins were identified, one encoded by Os10g0481500 (band A) and the other by Os05g0519000 (band C). The band 7 family, also known as the SPFH family, is a well-conserved superfamily that includes the FLOTILLIN (FLOT), prohibitin, stomatin, ER-linked lipid raft protein, and plant-specific HYPERSENSITIVE-INDUCED REACTION (HIR) subfamilies (Supplemental Fig. S2). Several members of this plant-specific subclass have been characterized as localized to PM microdomains and responsive to disease and osmotic stresses (Nohzadeh Malakshah et al., 2007; Jung et al., 2008; Yu et al., 2008; Zhou et al., 2009, 2010; Choi et al., 2011; Qi et al., 2011).

From band B, which was significantly increased in OX cells, two homologous proteins, annotated as ricin B-related lectin domain-containing proteins and encoded by Os03g0327600 and Os07g0683900, were identified (Supplemental Table S3). These proteins have also been called r40c1 and r40g2, respectively, and were first characterized as induced by osmotic stress and abscisic acid (Moons et al., 1997; Kawasaki et al., 2001). r40c1 was previously found in the rice DRM proteome (Fujiiwara et al., 2009); however, other proteomic studies have indicated various localizations of r40c1 and r40g2, such as the cytosol (Takahashi et al., 2005), cis-Golgi (Asakura et al., 2006), PM (Cheng et al., 2009), and even phloem sap (Aki et al., 2008). Fluorescence microscopy of GFP-tagged proteins supported the cytosolic localization of r40c1 (Supplemental Fig. S3), indicating that these proteins could be present ubiquitously in various cellular compartments, including PM microdomains.

**Figure 2.** SDS-PAGE profile of DRM fractions. Proteins (3 μg) of total membrane (TM) and DRM fractions prepared from PM were separated by SDS-PAGE and then silver stained. Arrowheads indicate significantly up- and down-regulated proteins in BI-1 OX cells. WT, Wild type.

**Table II.** Proteins with different abundances in wild-type and BI-1 OX cells

| Locus                     | Annotation                                      | Unique Peptides | Mean Ratio (OX:Wild Type) | SD |
|---------------------------|-------------------------------------------------|-----------------|---------------------------|----|
|                           |                                                  | Wild Type       | OX Compared a             |    |
| Up-regulated in OX cells  |                                                  |                 |                           |    |
| Os06g0680700               | Cytochrome P450 family protein                   | 1               | 1                         | 1  |
| Os07g0191200               | Plasma membrane H⁺-ATPase                         | 13              | 16                        | 13 |
| Os03g0327600               | Ricin B-related lectin domain-containing protein (r40c1) | 13              | 13                        | 13 |
| Os07g0683900               | Ricin B-related lectin domain-containing protein (r40g2) | 14              | 15                        | 14 |
| Os06g0113900               | Conserved hypothetical protein                    | 3               | 4                         | 3  |
| Os01g0834500               | Similar to 40S ribosomal protein S23             | 1               | 1                         | 1  |
| Os07g0194000               | Similar to VESICLE-ASSOCIATED MEMBRANE PROTEIN725 (AtVAMP725) | 3               | 4                         | 3  |
| Os03g0751100               | Similar to Isp4 protein-like                     | 3               | 4                         | 3  |
| Down-regulated in OX cells |                                                  |                 |                           |    |
| Os10g0481500               | Band 7 family protein (FLOT)                     | 11              | 1                         | 1  |
| Os08g0127100               | Amino acid transporter, transmembrane family protein | 3               | 1                         | 1  |
| Os07g0645000               | Allergen V5/Tpx-1-related family protein         | 1               | 1                         | 1  |
| Os05g0091900               | Band 7 family protein, similar to HIR3           | 8               | 7                         | 7  |
| Os08g0412700               | Unknown function DUF1262 family protein          | 4               | 2                         | 2  |
| Os10g0464000               | Band 7 family protein, similar to HIR5           | 5               | 3                         | 3  |
| Os07g0531500               | HARPIN-INDUCED1 domain-containing protein        | 2               | 2                         | 2  |
| Os03g0721200               | Armadillo-like helical domain-containing protein  | 1               | 1                         | 1  |
| Os08g0398400               | Band 7 family protein, similar to HIR1           | 6               | 5                         | 5  |

aFor detailed information, see Supplemental Table S4. ND, Not determined.
Shotgun Profiling and Comparative Analysis of DRM Proteomes

Gel-based comparative analysis demonstrated that BI-1 significantly alters several major DRM proteins in rice cells. We performed one-dimensional-PAGE/LC-MS/MS-based shotgun analysis to evaluate BI-1-mediated changes in a comprehensive DRM proteome, which identified 398 DRM proteins with at least one unique (gene-specific) peptide (for comprehensive proteome data and general analyses, see Supplemental Data Set S1, Supplemental Information S1, and Supplemental Table S6). Based on a label-free comparative analysis (Hamamoto et al., 2012), eight up-regulated and nine down-regulated proteins in OX cells were identified (Table II; all spectra with MS intensities are shown in Supplemental Table S4). The four proteins identified by the gel-based comparison described above (FLOT, HIR3, r40c1, and r40g2) were again included in this list with significant changes in mean ratio. In particular, substantially low values of the mean ratio were observed for FLOT and HIR3, indicating that the decreases in these band 7 family proteins, which were detected as bands with weak intensities (Fig. 2), were due to absolute loss of the molecules in the DRM rather than to a size shift on gels caused by posttranslational modification or partial degradation.

Intriguingly, the comparative analysis indicated that two additional HIR family proteins, HIR1 (Os08g0398400) and HIR5 (Os10g0464000), were moderately down-regulated in OX cells. Furthermore, one HAIRPIN-INDUCED1 (HIN1) domain-containing protein (Os07g0531500) was also reduced in OX cells; NONRACE-SPECIFIC DISEASE RESISTANCE1/HIN1-like family proteins are generally involved in a wide range of disease resistance responses, including cell death induced by the hypersensitive response. Although further experiments are necessary to confirm quantitative changes in the abundance of these death- or defense-related proteins in the DRM of BI-1 OX cells, this result implies that BI-1 OX alters a wide range of DRM proteins associated with stress responses.

Levels of FLOT and HIR3 in DRM of BI-1 OX Rice Cells Decrease without Transcriptional Changes

Among the proteins identified above, FLOT and HIR3, two major and markedly decreased DRM proteins in BI-1 cells, were further analyzed with respect to quantitative alterations and implication in the cell death tolerance phenotype of BI-1 OX cells. First, to validate the altered protein levels observed in comparative proteomics, we analyzed western blots of microsomal fractions. As shown in Figure 3A, both proteins were specifically detected in the PM and highly enriched in the DRM of wild-type cells, whereas no or low signals were obtained from the same protein load of microsomal membranes, even in detergent-resistant fractions. In addition, immunosignals of FLOT and HIR3 were substantially reduced in both the DRM and PM fractions of OX cells (Fig. 3A), confirming the results of MS-based comparative analyses. We also examined the subcellular localization of FLOT and HIR3 by confocal microscopy of fluorescently tagged proteins expressed transiently in onion (Allium cepa) epidermal cells (Fig. 3B). The fluorescence signals of FLOT and HIR3 were clearly colocalized with those of a PM marker (OSA7, a homolog of PM H+-ATPase encoded by Os04g0656100). After plasmolysis with 0.75 M mannitol, the fluorescence signals of these proteins were localized predominantly in the PM of shrunken protoplasts and also on Hechtian strands, PM extensions associated with the cell wall (Supplemental Fig. S4). These results collectively support the PM-specific localization of these proteins.

To determine whether the observed changes in DRM proteins were accompanied by changes in transcriptional regulation, the levels of FLOT and HIR3 transcripts were compared in wild-type and OX cells by quantitative reverse transcription (RT)-PCR. Despite the markedly low FLOT and HIR3 protein contents, the levels of their transcripts were not significantly different in wild-type and OX cells (Supplemental Fig. S5).

Roles of FLOT and HIR3 in Oxidative Stress- and SA-Induced Cell Death

Plant HIR family proteins are probably involved in cell death during responses to biotic and abiotic stresses.

![Figure 3. Subcellular localization of FLOT and HIR3. A, Western-blot analysis of FLOT and HIR3 in total membrane (TM) and DRM fractions of microsomes and PM prepared from wild-type (WT) and BI-1 OX cells. Silver-stained gel images corresponding to each protein are shown with comparable loading (5 μg of protein). B, Subcellular localization of red fluorescent protein (RFP)-tagged FLOT and HIR3 in onion epidermal cells (left images). Yellow fluorescent protein (YFP)-tagged rice PM H+-ATPase isof orm 7 (OSA7) was used as a PM marker (middle images). Right images are merged images of fluorescence and differential interference contrast (DIC).](Image)
In addition, low FLOT levels could affect the state of microdomain-resident proteins and protein complexes if the molecular functions of mammalian homologs are conserved in their plant counterparts. To assess whether the low protein levels of FLOT and HIR3 play a role in the enhanced stress tolerance of BI-1 OX rice cells, we analyzed loss-of-function insertional mutants. The insertion and mRNA expression of two tagged lines (flot and hir3) were analyzed by genomic PCR and sequencing, which verified that flot possesses a transfer DNA insertion within the first exon and that hir3 has a retrotransposon Tos17 insertion within the third intron (Fig. 4A), resulting in the absence of expression of the intact mRNA due to the insertion in each T3 homozygous line (Fig. 4B). Furthermore, we independently generated RNAi-mediated constitutive knockdown rice lines for FLOT and HIR3. RT-PCR confirmed significantly low transcript levels in the T1 generation (Fig. 4B). Using suspension cells derived from the mutants and knockdown lines, we assessed tolerance to menadione-mediated oxidative stress. Menadione treatment results in an immediate increase in reactive oxygen species in rice cells accompanied by the induction of cell death, which is effectively attenuated by overexpression of BI-1 (Ishikawa et al., 2010). After treatment of suspension cells with menadione, induced cell death was monitored by Evans blue dye uptake into dead cells. As shown in Figure 4, C and D, menadione-induced cell death was significantly attenuated in mutant and RNAi cells for both FLOT and HIR3. The attenuation of cell death was also observed when cells were treated with SA. Previous studies found that BI-1 expression is induced by SA treatment and that BI-1 overexpression increases the viability of rice cells after exposure to SA (Matsumura et al., 2003; Kawai-Yamada et al., 2004). In particular, the hir3 mutant and HIR3 RNAi cells exhibited strong suppression of SA-induced cell death (Fig. 4F). Knockout and knockdown lines for FLOT also showed a slight but statistically significant decrease in SA-induced cell death compared with wild-type cells (Fig. 4E). These results indicate that FLOT and HIR3 play a key role in oxidative stress- and SA-induced cell death via PM.
microdomains and that the low protein levels of FLOT and HIR3 could contribute directly to the stress-tolerant phenotype of BI-1 OX cells.

DISCUSSION

BI-1 interacts with FAH via the electron donor cytochrome b₅, which promotes 2-HFA production in Arabidopsis (Nagano et al., 2009, 2012). Moreover, ectopically expressed BI-1 fails to suppress BAX-induced cell death in a S. cerevisiae mutant lacking ScFAH1, suggesting that 2-HFAs are essential for BI-1-mediated cell death suppression. Our recent study demonstrated that AtFAH1 knockdown plants with a lower content of 2-hydroxy very-long-chain fatty acids (VLCFAs) are hypersensitive to oxidative stress (Nagano et al., 2012). As verified by comprehensive sphingolipidomics by LC-MS/MS (Markham et al., 2006), 2-hydroxy VLCFAs are typical components of plant sphingolipids. Thus, BI-1 and FAH seem to coordinate the maintenance of 2-hydroxylation of sphingolipids in the context of defense responses to oxidative stress. Furthermore, it was recently shown that Arabidopsis BI-1 interacts with other sphingolipid-modifying enzymes and is involved in sphingolipid metabolism under cold stress (Nagano et al., 2014). In this study, we confirmed that AtBI-1 overexpression resulted in the accumulation of both the predominant 2-hydroxy VLCFA species, 20h:0, and the total amount of GlcCer (Fig. 1). Thus, the BI-1 function that serves to promote the synthesis of 2-hydroxy VLCFAs is conserved in both Arabidopsis and rice, although the particular increase in 2-HFA species seems to differ in these plants, in which the molecular composition of HFA species naturally differs in length and degree of unsaturation. Furthermore, rice sphingolipids contain diunsaturated long-chain bases as their backbones, whereas Arabidopsis vegetative tissues contain only monounsaturated bases (Imai et al., 1997; Markham et al., 2006). These differences in fatty acid and long-chain base components might lead to the observed lower enrichment of GlcCer in rice compared with that reported for other plant materials. Further biochemical characterization of various plant sphingolipids will help to understand their behavior as membrane lipids in the formation of specific domains.

The involvement of sphingolipids in plant stress tolerance and cell death regulation is gradually being revealed: an enhanced cell death phenotype was observed in Arabidopsis mutants deficient in ceramide phosphorylation (Greenberg et al., 2000; Liang et al., 2003), sphingosine transfer protein (Brodersen et al., 2002), inositol phosphoceramide biosynthesis (Wang et al., 2008), and the hydroxylation of sphingoid long-chain bases (Chen et al., 2008) and fatty acyl chains (König et al., 2012; Nagano et al., 2012). These insights strongly support the importance of sphingolipids in plant stress tolerance and cell death regulation. However, there is little or no information on the mode of action of sphingolipids in the cell death regulatory pathway in plants.

In this study, we focused on PM microdomains as an intermediary between sphingolipids and cell death signaling, in which sphingolipids serve as basal membrane components in the formation of microdomain structures (Mongrand et al., 2004; Borner et al., 2005). DRM prepared from BI-1 OX rice cells exhibited higher GlcCer enrichment (Table I; Supplemental Fig. S1C) and different protein profiles in SDS-PAGE (Fig. 2; Supplemental Fig. S1D). The BI-1-mediated proteomic change in DRM was further analyzed by shotgun proteomics, which identified several up- and down-regulated proteins in BI-1 OX cells. Among the altered proteins, we focused on two band 7 family proteins, FLOT and HIR3. These two proteins were major DRM components, visible as clear bands following SDS-PAGE, and were diminished in DRM of BI-1 OX cells (Fig. 2). Quantitative decreases in FLOT and HIR3 levels were confirmed by comparative shotgun proteomics (Table II) and western blotting (Fig. 3). Further analysis using knockout and knockdown rice cells demonstrated that loss of FLOT and HIR3 caused the attenuation of cell death following exposure to oxidative stress and SA (Fig. 4). These proteins were specifically localized at PM-derived DRM (Fig. 3), suggesting that the cell death regulatory pathway responsive to oxidative stress and SA is localized in PM microdomains, in which FLOT and HIR3 play an indispensable role. This also implies that loss of these proteins from the microdomains is a key factor for enhanced stress tolerance in BI-1 OX cells.

HIR proteins are classified into the plant-specific clade of the band 7 family, and many homologous genes are conserved in plant species (Supplemental Fig. S2). HIRs were first identified in screens for homology to the tobacco NG1 peptide, which causes spontaneous formation of hypersensitive response-like lesions when ectopically overexpressed (Karrer et al., 1998; Nadimpalli et al., 2000). Overexpression of HIRs similarly leads to a

![Figure 5. Model for BI-1-mediated cell death suppression through modulating PM microdomain components. BI-1 overexpression causes sphingolipid-mediated domain remodeling or an unknown mechanism regulating microdomain proteins, leading to the exclusion of FLOT and HIR3 from these domains. The loss of these band 7 family proteins, which are orthologs of mammalian lipid raft-scaffolding proteins involved in signaling complex assembly, might cause the dissociation and inactivation of cell death-inducing machinery localized at the microdomains, which contributes to the stress-tolerant phenotype of BI-1 OX plants.](image-url)
lesion-mimic phenotype without pathogen challenge, indicating that they function as positive regulators of cell death induction (Jung and Hwang, 2007; Zhou et al., 2010). In addition, HIR overexpression enhances sensitivity to osmotic stress (Jung et al., 2008), suggesting that HIRs are involved in a variety of environmental stresses.

In contrast to the plant-specific HIR subfamily, FLOT is highly conserved in both plants and animals. Mammalian FLOT is a well-known lipid raft marker that serves as a major scaffolding molecule for lipid rafts by interacting with various proteins on the membrane microdomains involved in signal transduction, endocytosis, and cytoskeleton rearrangement (Langhorst et al., 2005). Although the molecular functions of plant FLOT are less known, the evolutionary conservation of this protein in animals and plants indicates that plant homologs have similar functions as a scaffold, suggesting that the presence of low levels of FLOT might lead to further changes in microdomain components, including signal transduction complexes. Interestingly, HIR homologs, members of the plant-specific band 7 subfamily distinct from FLOT, were recently reported to interact with membrane-associated signal transduction components and to be essential in cell death induction through the pathogen defense pathway (Jung and Hwang, 2007; Zhou et al., 2009; Qi et al., 2011). These findings suggest a working hypothesis, as shown in Figure 5, that loss of FLOT, HIR3, or both leads to changes in associated proteins involved in downstream functions such as cell death induction. Further analysis of the factors that interact with FLOT and HIR3 should advance our understanding of the cell death regulatory pathway via PM microdomains. In addition to these major DRM proteins, our proteomic data include several minor proteins with different abundances in BI-1 OX cells, which might be candidates for involvement in microdomain-localized regulatory complexes.

Although we show here evidence that sphingolipid content and composition were altered in PM microdomains of BI-1 OX cells, these changes were not as drastic as the quantitative changes in several DRM proteins. Thus, further studies are necessary to address the causal relationships and molecular mechanisms involved in the alterations of sphingolipids and DRM proteins in BI-1 OX cells. Generation and biochemical analyses of transgenic plants with altered levels of BI-1 and/or GlcCer will provide causal relationships between the composition of sphingolipids and proteins in PM microdomains. Another interesting issue remaining for future studies is how FLOT and HIR3 decline in BI-1 OX cells. Quantitative RT-PCR revealed comparable transcript levels of FLOT and HIR3 in wild-type and BI-1 OX cells, suggesting that the decrease in these proteins were posttranscriptionally regulated. In our proteomic and western-blot analyses, no increase in FLOT or HIR3 was detected in other membrane fractions that compensated for their decrease in PM-DRM fractions. These proteins do not possess any possible transmembrane domains but are anchored to membranes via several acyl modifications (Neumann-Giesen et al., 2004; Jung and Hwang, 2007), suggesting that these proteins might move to other compartments as soluble proteins.

In conclusion, our proteomic approach demonstrated that changes in the DRM protein profile, especially decreases in the levels of FLOT and HIR3 proteins, occurred in BI-1 OX cells. Loss-of-function analysis demonstrated that FLOT and HIR3 modulate cell death induced by oxidative stress and SA, suggesting a cell death-inducing pathway through PM microdomains dependent on the putative scaffolding proteins FLOT and HIR3. These findings also provide a working hypothesis for the mechanistic roles of sphingolipids and membrane microdomains in the stress-tolerant phenotype of BI-1 OX plants (Fig. 5).

MATERIALS AND METHODS

Rice Cell Culture

Suspension cells of wild-type and BI-1 OX rice (*Oryza sativa* ‘Sasanishiki’) were prepared and maintained in Chu’s N6 medium as described previously (Matsumura et al., 2003; Ishikawa et al., 2010). Four-day-old cells were used for analyses.

2-HFA Analysis

GlcCer was prepared as described previously (Minamioka and Imai, 2009). Fatty acids were liberated as methyl esters and analyzed by GC-MS according to Nagano et al. (2009).

Membrane Preparation

Rice cells were homogenized in 2.5 volumes of extraction buffer (25 mM Tris-MES, pH 7.5, containing 5 mM EDTA, 2.5 mM dithiothreitol [DTT], 0.25 mM Suc, and 0.6% [w/v] polyvinylpyrrolidone) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 mM leupeptin, and 0.2 mM pepstatin) using a Polytron homogenizer. The homogenate was sequentially centrifuged at 1,000g for 10 min, 10,000g for 20 min, and 100,000g for 30 min at 4°C. The 100,000g pellet (microsomal membrane fraction) was washed once and resuspended in partition buffer (10 mM potassium phosphate, pH 7.6, 0.25 mM Suc, 30 mM NaCl, 1 mM EDTA, and 0.1 mM DTT) followed by aqueous two-phase partitioning with 6% (w/w) polyethylene glycol 3350/dextran 500 in partition buffer. Partitioning was repeated three times, and the final upper layer was centrifuged (100,000g, 30 min, and 4°C) after dilution with Tris-buffered saline (TBS). The pellet was washed once and resuspended in TBS containing 1 mM DTT and the protease inhibitors (PM fraction). The PM fraction was treated with 1% (v/v) Triton X-100 (protein: detergent ratio, 1:15) for 30 min on ice. To the lysate, a solution of Suc in TBS was added to 48% (w/w) Suc, and this mixture was layered with 40%, 35%, 30%, and 5% (w/v) Suc and centrifuged at 200,000g for 16 h at 4°C. DRM was collected as an off-white ring above the 30% to 35% interface, diluted with TBS, and pelleted by centrifugation. The pellet was resuspended in TBS supplemented with 1 mM DTT and protease inhibitors and stored at −80°C until use.

Protein Quantitation and Western-Blot Analysis

The protein concentration of membrane fractions was determined with a bicinchoninic acid protein assay kit (Pierce) using bovine serum albumin as a standard after a solubilization with 2% (w/v) SDS. Rabbit polyclonal anti-FLOT antibody was raised against a mixture of two synthetic oligopeptides, C-ARQREAELYAKQKEA (351–367) and C-PAWMGLTGGAPSSTS (470–485; C terminal). To obtain a HIR3-specific antibody, the C-terminal residues of HIR3 (C-RDGLLQQAITTS; 275–288) were used as antigen, with the Cys residues at the N terminus of each peptide added for carrier conjugation. Peptide synthesis, antibody preparation, and affinity purification were performed by Operon Biotechnology on consignment. Markers of membrane fractions were analyzed as follows: PM, anti-Arabidopsis (*Arabidopsis thaliana*) PM

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Marker Enzyme Assay

Marker enzyme assays of membrane fractions were performed as follows: vanadate-sensitive ATPase for PM (Sandstrom et al., 1987), NO3-sensitive ATPase for tonoplast (O'Neill et al., 1983), azide-sensitive ATPase for mitochondria (Gallagher and Leonard, 1982), IDPase for Golgi (Mitsui et al., 1994), and NADH-cytochrome c reductase for ER (Hodges and Leonard, 1974).

LC-MS/MS Shotgun Analysis

Membrane fractions were incubated in Laemmli buffer (100 mM DTT) at 70°C for 10 min, separated by SDS-PAGE, stained with Coomassie Brilliant Blue, and sliced into 10 pieces. Each slice was digested in gel and then analyzed by LC-MS/MS using a nano-flow HPLC chip system coupled with a mass spectrometer (Agilent Technologies) as described previously (Aki et al., 2008). The spectra obtained were searched against the Rice Annotation Project Database using Spectrum Mill software (Agilent Technologies). MS intensity-based semiquantitative analysis was performed as described in detail previously (Hamamoto et al., 2012). Proteins with a mean ratio greater than 3-fold were considered different in abundance.

RT-PCR Analysis

RNA preparation and complementary DNA synthesis were performed as described previously (Ishikawa et al., 2010). Quantitative PCR was conducted with the Power SYBR Green PCR Master Mix and a 7300 real-time PCR system (Applied Biosystems). Primer sequences are shown in Supplemental Table S5.

Microscopic Examination

Cauliflower mosaic virus 35S promoter-driven FLOT-RFP, HIR3-RFP, and YFP-OSA7 (PM H+-ATPase; OsH4g0656010) were transiently expressed in onion (Allium cepa) epidermal cells following particle bombardment. Confocal laser scanning microscopy was performed using an FV-1000 microscope (Olympus).

Insertional Mutants and RNAi Transgenics

Transfer DNA (Tob and Toss1 (hir)) insertional mutants were obtained from the Oryza Tag Line database (Droc et al., 2006). Homozygous mutant lines were determined by genomic PCR and used for the analyses described below. RNAi vectors were constructed using pANDA (Miki and Shimamoto, 2004) with the partial coding sequence; FLOT vectors were constructed using pANDA by LR Clonase (Invitrogen). Cloned into the pENTR-D/TOPO or pDONR207 vector (Invitrogen), followed by transfer to pANDA by LR Clonase (Invitrogen). Agrobacterium tumefaciens containing constructs were introduced into the rice cv. Nipponbare for T-DNA insertion. Homozygous mutant lines were obtained from wild-type plants after selection with hygromycin B.

Cell Death Assay

Oxidative stress was induced by treatment of 4-d-old cells with 400 μM menadione in N6 medium. SA was added to medium at a final concentration of 500 μM. Chemicals were dissolved in dimethyl sulfoxide at 500× concentration. Cell death was assessed by Evans blue uptake 24 h after treatment as described previously (Ishikawa et al., 2010).

The accession numbers of the rice genes cited in this article can be found in the Rice Annotation Project Database and the KOME (Knowledge-based Oryza Molecular biological Encyclopedia) database.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Characterization of membrane fractions prepared from BI-1 OX rice cells.

Supplemental Figure S2. Phylogenetic tree of band 7 family proteins.

Supplemental Figure S3. Subcellular localization of s40c1 in onion epidermal cells.

Supplemental Figure S4. FLOT and HIR3 localization in plasmolyzed onion epidermal cells.

Supplemental Figure S5. Quantitative RT-PCR analysis of DRM proteins.

Supplemental Table S1. Fatty acid composition of GlcCer fractions from wild-type and BI-1 OX rice cells.

Supplemental Table S2. Marker enzyme activity of membrane fractions from wild-type and BI-1 OX rice cells.

Supplemental Table S3. LC-MS/MS analysis of proteins with visible differences in SDS-PAGE profiles between wild-type and OX cells.

Supplemental Table S4. Peptides used for comparative analysis.

Supplemental Table S5. Primer sequences used in this study.

Supplemental Table S6. Functional categorization and quantitative comparison of DRM proteins.

Supplemental Information S1. Additional results and methods.

Supplemental Data Set S1. DRM proteins identified in this study.

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LITERATURE CITED

Aki T, Shigyo M, Nakano R, Yoneyama T, Yanagisawa S (2008) Nano scale proteomics revealed the presence of regulatory proteins including three FT-Like proteins in phloem and xylem saps from rice. Plant Cell Physiol 49: 767–780

Asakura T, Hirose S, Katamine H, Kitajima A, Hori H, Sato MH, Fujiwara M, Shimamoto K, Mitsui T (2006) Isolation and proteomic analysis of rice Golgi membranes: cis-Golgi membranes labeled with GFP-SYP31. Plant Biotechnol 23: 475–485

Bomer GH, Sherrer DR, Weimar T, Michaelson LV, Hawkins ND, Macaskill A, Napier JA, Beale MH, Lilley KS, Davenport P (2005) Analysis of detergent-resistant membranes in Arabidopsis: evidence for plasma membrane lipid rafts. Plant Physiol 137: 104–116

Brodersen P, Petersen M, Pike HM, Olszak B, Skov S, Ødum N, Jorgensen LB, Brown RE, Mundy J (2002) Knockout of Arabidopsis hir3, a leucine-rich repeat protein, causes a loss of cell death associated with plant cell death. Genes Dev 16: 490–502

Chen M, Markham JE, Caahoon EB (2012) Sphingolipid Δ8 unsaturation is important for glucosylceramide biosynthesis and low-temperature performance in Arabidopsis. Plant J 69: 769–781

Chen M, Markham JE, Dietrich CR, Jaworski JG, Caahoon EB (2008) Sphingolipid long-chain base hydroxylation is important for growth and regulation of sphingolipid content and composition in Arabidopsis. Plant Cell 20: 1862–1878

Cheng Y, Qi Y, Zhu Q, Chen X, Wang N, Zhao X, Chen H, Cui X, Xu L, Zhang W (2009) New changes in the plasma-membrane-associated proteome of rice roots under salt stress. Proteomics 9: 3100–3114

Choi HW, Kim YJ, Hwang BK (2011) The hypersensitive induced reaction and leucine-rich repeat proteins regulate plant cell death associated with disease and plant immunity. Mol Plant Microbe Interact 24: 68–78
König S, Feusner K, Schwarz M, Kaever A, Iven T, Landesfeind M, Ternes P, Karlovsky P, Lipka V, Feusner I (2012) Arabidopsis mutants of sphingolipid fatty acid α-hydroxylases accumulate ceramides and salicylates. New Phytol 196: 1086–1097

Langhorst MF, Reuter A, Steuerer CA (2005) Scaffolding microdomains and beyond: the function of reggie/flotilin proteins. Cell Mol Life Sci 62: 2228–2240

Li R, Liu P, Pan Y, Chen T, Wang Q, Mettbach U, Baluska F, Samaj J, Fang X, Lucas WJ, et al (2012) A membrane microdomain-associated protein, Arabidopsis Flo1, is involved in a clathrin-independent endocytic pathway and is required for seedling development. Plant Cell 24: 2105–2122

Liang H, Yao N, Song JT, Luo S, Lu H, Greenberg JT (2003) Ceramides modulated programmed cell death in plants. Genes Dev 17: 2636–2641

Liu F, Li RL, Zhang L, Wang QL, Niehaus K, Baluska F, Samaj J, Lin JX (2009) Lipid microdomain polarization is required for NADPH oxidase-dependent ROS signaling in Picea mariana pollen tube tip growth. Plant J 60: 303–313

Markham JE, Li J, Cahoon EB, Jaworski GD (2006) Separation and identification of major plant sphingolipid classes from leaves. J Biol Chem 281: 22684–22694

Markham JE, Molino D, Gissot L, Bellec Y, Hématy K, Marion J, Belcram K, Bouchez OJ, Salat-Jeunemaître B, Faure JD (2011) Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in Arabidopsis. Plant Cell 23: 2362–2378

Matsumura H, Nirasawa S, Kiba A, Urassak N, Saitoh H, Ito M, Kawai-Yamada M, Uchimiya H, Terauchi R (2003) Overexpression of Bax inhibitor suppresses the fungal-and-elicitor-induced cell death in rice (Oryza sativa L). Plant J 33: 425–434

Melser S, Bataille B, Peypelut M, Poujol C, Bellec Y, Wattleboyer Y, Manetta-Peyret L, Faure JD, Moreau P (2010) Glucosylceramide biosynthesis is involved in Golgi morphology and protein secretion in plant cells. Traffic 11: 479–490

Milks D, Shimamoto K (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant Cell Physiol 45: 490–499

Milbas D, Clarke CJ, Hannun YA (2010) Sphingomyelin metabolism at the plasma membrane: implications for bioactive sphingolipids. FEBS Lett 584: 1887–1894

Minami A, Fujimura M, Furuto A, Kamo M, Kawamura Y, Uemura M (2009) Alterations in detergent-resistant plasma membrane microdomains in Arabidopsis thaliana during cold acclimation. Plant Cell Physiol 50: 341–359

Minamioka H, Imai H, Markham JE, Molino D, Gissot L, Bellec Y, Hématy K, Marion J, Belcram K, Bouchez OJ, Salat-Jeunemaître B, Faure JD (2011) Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in Arabidopsis. Plant Cell 23: 2362–2378

Markham JE, Molino D, Gissot L, Bellec Y, Hématy K, Marion J, Belcram K, Bouchez OJ, Salat-Jeunemaître B, Faure JD (2011) Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in Arabidopsis. Plant Cell 23: 2362–2378

Mitsui T, Honma N, Kondo T, Hashimoto N, Kimura S, Igaue I (1994) Structure and function of the Golgi complex in rice cells: II. Purification and characterization of Golgi membrane-bound nucleoside diphosphatase. Plant Physiol 106: 351–359

Monteagudo S, Morel J, Larche J, Claverol S, Carde JP, Hartmann MA, Bonneu M, Simon-Plas F, Lessire R, Bessoule J (2004) Lipid rafts in higher plant cells: purification and characterization of Triton X-100-insoluble microdomains from tobacco plasma membrane. J Biol Chem 279: 36277–36286

Moons A, Gielen J, Vandenverckhove J, Van der Staetzen D, Gheysen G, Van Montagu M (1997) An abscisic-acid- and salt-stress-responsive rice cDNA from a novel plant gene family. Plant Physiol 120: 443–454

Mortimer JC, Yu X, Albrecht S, Sicilia F, Huichalaf M, Ampuero D, Michaelson LV, Murphy AM, Matsunaga T, Kurz S, et al (2013) Abnormal glycosphingolipid mannosylation triggers salicylic acid-mediated responses in Arabidopsis. Plant Cell 25: 1881–1894

Nadimpalli R, Yalpani N, Holaj GS, Simmons CR (2000) Prohibitins, stomatins, and plant disease response genes compose a protein superfamily that controls cell proliferation, ion channel regulation, and death. J Biol Chem 275: 29579–29586

Nagano M, Ibarra-Ohori Y, Imai H, Inada N, Fujimoto M, Tsutsui N, Uchimiya H, Kawai-Yamada M (2009) Functional association of cell death suppressor Arabidopsis Bax inhibitor-1, with fatty acid 2-hydroxylations through cytochrome bs. Plant J 58: 122–134

Nagano M, Ishikawa T, Ogawa Y, Iwabuchi M, Nakasone A, Shimamoto K, Uchimiya H, Kawai-Yamada M (2014) Arabidopsis Bax inhibitor-1 promotes sphingolipid synthesis during cold stress by interacting with ceramide-modifying enzymes. Plant 240: 77–89
Nagano M, Takahara K, Fujimoto M, Tsutsuimi N, Uchimiya H, Kawai-Yamada M (2012) Arabidopsis sphingolipid fatty acid 2-hydroxylases (AtFAH1 and AtFAH2) are functionally differentiated in fatty acid 2-hydroxylation and stress responses. Plant Physiol 159:1138–1148

Neumann-Giesen C, Falkenbach B, Beicht P, Claasen S, Lüers G, Steuermer CAO, Herzog V, Tikkanen R (2004) Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. Biochem J 378:509–518

Ng CKY, Carr K, McAinsh MR, Powell B, Hetherington AM (2001) Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. Nature 410:596–599

Nohzadeh Malakshah S, Habibi Rezaei M, Heidari M, Salekdeh GH (2007) Characterization of a NO3-sensitive H+-ATPase from corn roots. Plant Physiol 72:837–846

Pata MO, Hannun YA, Ng CK (2010) Plant sphingolipids: decoding the enigma of the Sphinx. New Phytol 185:611–630

Peskan T, Westermann M, Oelmüller R (2000) Identification of low-density Triton X-100-insoluble plasma membrane microdomains in higher plants. Eur J Biochem 267:6989–6995

Qi Y, Tsuda K, Nguyen V, Wang X, Lin J, Murphy AS, Glazebrook J, Thordal-Christensen H, Katagiri F (2011) Physical association of Arabidopsis hypersensitive induced reaction proteins (HIRs) with the immune receptor RPS2. J Biol Chem 286:31297–31307

Sanchez P, de Torres Zabala M, Grant M (2000) AtBI-1, a plant homologue of Bax inhibitor-1, suppresses Bax-induced cell death in yeast and is rapidly upregulated during wounding and pathogen challenge. Plant J 21:393–399

Sandstrom RP, Deboer AH, Lomax TL, Cleland RE (1987) Latency of plasma membrane H+-ATPase in vesicles isolated by aqueous phase partitioning: increased substrate accessibility or enzyme activation. Plant Physiol 85:693–698

Saucedo-García M, Guevara-García A, González-Solís A, Cruz-García F, Vázquez-Santana S, Markham JE, Lozano-Rosas MG, Dietrich CR, Ramos-Vega M, Cahoon EB, et al (2011) MPK6, sphinganine and the LCB2a gene from serine palmitoyltransferase are required in the signaling pathway that mediates cell death induced by long chain bases in Arabidopsis. New Phytol 191:943–957

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31–39

Staubach S, Hanisch FG (2011) Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. Expert Rev Proteomics 8:263–277

Takahashi H, Hotta Y, Hayashi M, Kawai-Yamada M, Komatsu S, Uchimiya H (2005) High throughput metabolome and proteome analysis of transgenic rice plants (Oryza sativa L.). Plant Biotechnol 22:47–50

Toki S, Hara N, Ono K, Onodera H, Tagiri A, Oka S, Tanaka H (2006) Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. Plant J 47:969–976

Townley HE, McDonald K, Jenkins GL, Knight MR, Leaver CJ (2005) Ceramides induce programmed cell death in Arabidopsis cells in a calcium-dependent manner. Biol Chem 386:161–166

Wang W, Yang X, Tangchaiburana S, Ndeh R, Markham JE, Tsegaye Y, Dunn TM, Wang GL, Bellizzi M, Parsons JF, et al (2008) An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in Arabidopsis. Plant Cell 20:3163–3179

Watanabe N, Lam E (2006) Arabidopsis Bax inhibitor-1 functions as an attenuator of biotic and abiotic types of cell death. Plant J 45:884–894

Watanabe N, Lam E (2008) BAX inhibitor-1 modulates endoplasmic reticulum stress-mediated programmed cell death in Arabidopsis. J Biol Chem 283:3200–3210

Xu Q, Reed JC (1998) Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. Mol Cell 1:337–346

Yu XM, Yu XD, Qu ZP, Huang XJ, Guo J, Han QM, Zhao J, Huang LL, Kang ZS (2008) Cloning of a putative hypersensitive induced reaction gene from wheat infected by stripe rust fungus. Gene 407:193–198

Zhou L, Cheung MY, Li MW, Fu Y, Sun Z, Sun SS, Lam HM (2010) Rice hypersensitive induced reaction protein 1 (OsHIR1) associates with plasma membrane and triggers hypersensitive cell death. BMC Plant Biol 10:290–299

Zhou L, Cheung MY, Zhang Q, Lei CL, Zhang SH, Sun SS, Lam HM (2009) A novel simple extracellular leucine-rich repeat (eLRR) domain protein from rice (OsLRR1) enters the endosomal pathway and interacts with the hypersensitive-induced reaction protein 1 (OsHIR1). Plant Cell Environ 32:1804–1820

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