The Arabidopsis ABHD11 Mutant Accumulates Polar Lipids in Leaves as a Consequence of Absent Acylhydrolase Activity\[OPEN\]

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Alpha/beta hydrolase domain (ABHD)-containing proteins are structurally related with diverse catalytic activities. In various species, some ABHD proteins have been characterized and shown to play roles in lipid homeostasis. However, little is known about ABHD proteins in plants. Here, we characterized AT4G10030 (ATABHD11), an Arabidopsis (Arabidopsis thaliana) homolog of a human ABHD11 gene. In silico analyses of ATABHD11 revealed homology with other plant species with a conserved GXSXG lipid motif. Interestingly, Arabidopsis abhd11 mutant plants exhibited an enhanced growth rate compared with wild-type plants. Quantitative analyses of the total lipids showed that the mutant abhd11 has a high amount of phospholipid and galactolipid in Arabidopsis leaves. The overexpression of ATABHD11 in Escherichia coli led to a reduction in phospholipid levels. The bacterially expressed recombinant ATABHD11 hydrolyzed lyso(phospho)lipid and monoacylglycerol. Furthermore, using whole-genome microarray and real-time PCR analyses of abhd11 and wild-type plants, we noted the up-regulation of MGD1, -2, and -3 and DGD1. Together, these findings suggested that ATABHD11 is a lyso(phospholipidase. The disruption of ATABHD11 caused the accumulation of the polar lipids in leaves, which in turn promoted a higher growth rate compared with wild-type plants.

Mutations in ABHD5/CGI58 cause Chanarin-Dorfman syndrome, an autosomal recessive disorder in humans (Rozenszajn et al., 1966; Dorfman et al., 1974; Chanarin et al., 1975). More recently, studies have demonstrated that human CGI-58 has lysophosphatidic acid (LPA) acyltransferase (Ghosh et al., 2008a) and lysophosphatidylglycerol (LPG) acyltransferase (Zhang et al., 2014) activities. In various cancers, high levels of ABHD11 mRNA transcripts have been reported, but the role of this enzyme in cancer metabolism is not known. ABHD11 has been identified as a possible biomarker for lung carcinoma (Wiedl et al., 2011). In vivo metabolite profiling revealed that human ABHD3 overexpressing cells exhibited elevated levels of phospholipase activity (Long et al., 2011). In a similar study, ABHD6 was shown to have an enzymatic activity that hydrolyzed both monoacylglycerol (MAG) and lysophospholipid (Thomas et al., 2013). In a rodent model, ABHD12 was reported to be a major lysophosphatidyl-Ser and MAG lipase (Blankman et al., 2013). Similarly, ABHD16A was shown to hydrolyze phosphatidyl-Ser (PS) in mammalian systems. Disruption of these two enzymes showed altered phospholipid and lysophospholipid levels and caused neuroimmunological disorders in mice (Kamat et al., 2015). ICT1, an ABHD6 homolog in yeast, was shown to encode a protein with soluble LPA acyltransferase activity that could enhance phospholipid synthesis during organic solvent stress (Ghosh et al., 2008b). Based on these studies, it is clear that ABHD proteins play a
major role in maintaining lipid homeostasis in eu-
karyotic organisms.

In Arabidopsis (*Arabidopsis thaliana*), ABHD is con-
sidered to be a large family of proteins. PES1 and PES2
from Arabidopsis belong to the ABHD family of pro-
teins, which have been shown to play a role in phytol
ester biosynthesis in chloroplasts (Lippold et al., 2012).
Recombinant Arabidopsis CGI58 was demonstrated to
possess LPA acyltransferase activity (Ghosh et al.,
2009). Moreover, in 2010, James et al. observed the ac-
cumulation of neutral lipids in the leaves of Arabi-
dopsis *cgi58* mutants.

**Figure 1.** In silico analyses of AtABHD11. A, Neighbor-joining
tree of AtABHD11 along with known plant ABHD11 protein
sequences. The maximum-likelihood bootstrap support is indicated in red
color. The bootstrap threshold was set to 70% and the confidence level
to over 95% as highlighted with bold lines. The scale bar indicates
the number of amino acid substi-
tutions per site. B, Sequence com-
parison of the AtABHD11 proteins
with other closely related plant
ABHD proteins. The species in-
clude G. soja, T. urartu, and G.
arboreum. Residues conserved in
all proteins are indicated using a
conservation graph. Red underline
shows the conserved lipid motifs in
plant ABHD11 proteins.
Herein, we attempted to understand the role of the human ABHD11 homolog in Arabidopsis. We used both transcriptomic and lipidomic approaches to characterize AT4G10030 (AtABHD11). In silico analyses were carried out to explore the sequence similarities of AtABHD11 with other plant species, as well as within the Arabidopsis genome. Lipidomics analyses revealed that disruption of abhd11 caused a significant increase in leaf phospholipids and galactolipids compared with the wild-type levels. Microarray profiling of abhd11 mutant plants showed the altered expression of genes involved in galactolipid and phospholipid metabolic pathways. In vitro biochemical studies revealed the presence of lyso(phospho)lipase and MAG lipase activities for heterologously overexpressed recombinant AtABHD11, demonstrating that it plays an important role in maintaining lipid homeostasis in Arabidopsis leaves.

RESULTS

AtABHD11 and Its Plant Homologs

To identify homologs of ABHD-containing proteins in Arabidopsis and other plant species, the protein sequence of AT4G10030 (AtABHD11) was retrieved from The Arabidopsis Information Resource database. Homology searches in Arabidopsis and other plant species revealed that 24 Arabidopsis proteins were homologous. However, ABHD11 proteins from other plant species, such as Triticum urartu,
Figure 2. Characterization of the abhd11 mutant line. A, Genomic organization of the T-DNA-inserted abhd11 deficient mutant. Black boxes indicate the exons, and the triangle indicates the site of T-DNA insertion in the AtABHD11 genome. The inset shows PCR amplification using genomic DNA from 3-week-old Columbia-0 and abhd11 plants. B, Real-time PCR analysis of the Arabidopsis ABHD11 mRNA expression levels in various tissues of Columbia-0. Graph shows the expression pattern of AtABHD11 as compared to rosette leaf stage. C, Real-time PCR analysis of AtABHD11 relative expression in 3-week-old leaves. Each value represents the mean ± SD of three biological replicates. Actin was used as an internal control to determine the quality and quantity of cDNAs. D, Phenotype of the abhd11 mutant at different growth stages as compared to Columbia-0 (wild-type). abhd11, salk_090222c.
Figure 3. Phospholipid changes in abhd11 by tandem electrospray ionization mass spectrometry. A, Phospholipid content of wild-type (WT) and abhd11 mutant plant leaves of Arabidopsis. B, Differences in PC, PG, PE, and PI molecular species. Asterisk shows an increase at $P < 0.05$ as compared with the respective wild-type signal. All values are means ± SD of three biological replicates. Molecular species are given as total acyl carbon:double bonds.
Gossypium arboreum, and Gly soja, showed the greatest homology with AtABHD11 (Fig. 1A). In 2009, Ghosh et al. demonstrated three different conserved motifs of Arabidopsis ABHD-containing proteins. A sequence comparison of AtABHD11 with closely related plant species showed commonality in the amino acid residues with a conserved lipase (GXGXG) motif. Additionally, these proteins also showed a conserved acyltransferase (HXXXXD) motif (Fig. 1B).

Identification of a Homozygous abhd11 Mutant and Phenotype Analysis

We investigated all of the SALK T-DNA insertion mutant lines of Arabidopsis ABHD11 that were

Figure 4. Altered galactolipid composition in abhd11. A, Overall increase in galactolipids in abhd11 compared with wild-type (WT) Arabidopsis leaves. B and C, Molecular species of MGDG (B) and DGDG (C); asterisk shows the increase at P < 0.05 compared with the respective wild-type signal. D, Analysis of the fatty acid content in the leaf TAG of abhd11 and wild-type plants. E, Analysis of seed TAG content of abhd11 and wild-type plants. All the values are the means (±sd) of three biological replicates. All lipid species are presented as total acyl carbon:double bonds.
available from the Arabidopsis Biological Resource Center. PCR was performed with genomic DNA extracted from Columbia-0 (wild-type) and SALK mutants of ABHD11. Homozygous lines were screened using T-DNA border-specific primers and the gene-specific primers as given in Table I. Among eight SALK lines, the presence of a T-DNA insertion was confirmed only in SALK_090222c (abhd11) by PCR (Fig. 2A, inset). The site of the DNA insertion was indicated by a triangle in a genomic DNA map. Black boxes and lines represent coding and noncoding regions of the AtABHD11 genome, respectively (Fig. 2A). In silico analysis by Genevestigator showed that the expression of ABHD11 was elevated right before bolting stage. Further validation was carried out by real-time PCR; the analysis revealed that the expression of ABHD11 was indeed high in the rosette leaves as compared to other stages in the wild type (Fig. 2B). Hence, the rosette stage was chosen to carry out the further experiments. To check the ABHD11 mRNA expression level in the mutant, quantitative real-time PCR was performed. Figure 2C shows the expression of AtABHD11 was more than 3-fold reduced compared with the wild type (Fig. 2C). Wild-type and abhd11 plants were grown on soil supplemented with one-half Murashige and Skoog medium under optimal conditions. The growth pattern of wild-type and abhd11 plants was analyzed at 3 weeks (Fig. 2D, inset), 6 weeks (Fig. 2D, left), and 10 weeks (Fig. 2D, right). The abhd11 plants showed an early bolting and higher rate of growth as compared with wild-type plants. The consistency of changes in the growth patterns was observed in plants from different batches. However, the size of the leaves in abhd11 remained the same as the control plants.

Altered Lipid Profile in abhd11

Total lipids were analyzed by tandem electrospray ionization mass spectrometry. Polar lipids (phosphatidic acid [PA], phosphatidylcholine [PC], phosphatidylethanolamine [PE], phosphatidylglycerol [PG], phosphatidylinositol [PI], PS, monogalactosyldiacylglycerol

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**Figure 5.** Heterologous overexpression of AtABHD11. A, SDS-PAGE (12%) of Ni²⁺-NTA-purified AtABHD11 expressed in E. coli cells. Empty vector was transformed into BL21 cells, and the cell-free extract was subjected to Ni²⁺-NTA column. The eluted fraction of vector background was used as a control. B, Immunoblot confirmation of recombinant AtABHD11. C, E. coli cells carrying pRSET-A-AtABHD11 and pRSET-A alone were induced with IPTG in the presence of 14C-acetate (0.5 μCi/mL). A_{600} = 20 (equivalent to 4 mg of dry weight) of induced cells were harvested, and lipids were extracted and separated on two-dimensional TLC using a phospholipid solvent system. The insets show the incorporation of labeled acetate into various phospholipids. Graph values represent the mean (±sd) of five independent experiments. CL, Cardiolipin.
[MGDG], and digalactosyldiacylglycerol [DGDG]) were analyzed from the leaves of both wild-type and abhd11 tissues. Triacylglycerol (TAG) was analyzed from the seeds and leaves of wild-type and abhd11 tissues. Although abhd11 disruption caused an overall increase in the phospholipid content, a significant increase was observed only in PC, followed by PE, PG, and PI, whereas PA and PS did not show any significant changes (Fig. 3A). Lipid molecular species identification of PC, PE, PG, and PI showed an increased amount of unsaturated fatty acids with 16:1, 18:2, and 18:3 (Fig. 3B). Similarly, galactolipids, such as DGDG and MGDG, also showed a significant increase in abhd11 mutant plants (Fig. 4A). The analyses of abhd11 plants for DGDG and MGDG showed an elevation in the levels of (34:6)MGDG and (36:6)MGDG (Fig. 4B) and (36:6)DGDG and (34:3)DGDG (Fig. 4C) compared with wild-type plants. Neutral loss scans for various acyl fatty acids of leaves and seeds TAG did not reveal any significant changes in the abhd11 mutant (Fig. 4D and 4E). In addition, TLC analysis for phospholipids of abhd11 mutant seeds had no profound changes when compared to the wild type. Together, lipidomics data for abhd11 suggest that AtABHD11 may play a role in phospholipid metabolism and could possibly channel phospholipids toward galactolipid generation.

**Recombinant AtABHD11 Alters Phospholipids in* Escherichia coli**

To test our hypothesis of an association of AtABHD11 in phospholipid metabolism, AtABHD11 was overexpressed in BL21 (DE3) cells, and the protein was purified from the soluble fraction using a Ni²⁺-NTA column from an overexpressed pRSET-A vector and AtABHD11 as control. C, lysophospholipase activity of AtABHD11. Both phospholipid and lysophospholipase activities were carried out with purified proteins from recombinant AtABHD11 and empty vector, and the assays were carried out for 30 min at 30°C. Assay contained 50 μM NBD-fluorescent fatty acid-labeled lysophospholipids substrates in the presence of 10 mM CaCl₂. Assay reactions were stopped, and lipids were extracted and separated on a silica-TLC plate using petroleum ether:diethyl ether:acetic acid (70:30:1, v/v) as the solvent system. D, The assay was performed with 50 μM [1-14C]MAG (0.025 μCi/tube). Lipids were extracted and then resolved on a silica-TLC plate using the solvent system described above. —ENZ, The assay was performed without enzyme; VEC, purified protein from overexpressed vector used as an enzyme; GENE, purified protein from overexpressed ABHD11 used as an enzyme. Graphs represent the mean (±SD) of three independent experiments.
Acylhydrolase Activity of AtABHD11

It was clear from the earlier experiment that AtABHD11 had a potential role in phospholipid metabolism. Accordingly, a significant reduction in phospholipid levels was observed when AtABHD11 was expressed heterologously. The presence of hydrolytic activity was analyzed using Ni²⁺-NTA purified AtABHD11 protein. In vitro assays were performed with total lipids as substrate, extracted from [¹⁴C]acetate-labeled wild-type yeast cells. As shown in Figure 6A, two-dimensional TLC analysis confirmed that AtABHD11 protein had the capacity to hydrolyze phospholipid more efficiently than the protein purified from the vector control. This experiment also showed the broad substrate specificity of the phospholipase activity. To validate further, the phospholipase activity of AtABHD11 was monitored with various individual lysophospholipids (LPA, lyso-phosphatidylcholine [LPC], lyso-phosphatidylethanolamine [LPE], LPG, lyso-phosphatidylinositol, and lyso-phosphatidylserine), phospholipids (PA, PC, PE, PG, PI, and PS), and nonpolar (MAG, diacylglycerol [DAG], and TAG) lipid substrates. All the assays were performed by incubating 2 µg of purified recombinant AtABHD11 with 50 µM of various substrates for 30 min at 30°C. The high sensitive fluorescent-based method was used to estimate the released free fatty acid (FFA).
from various substrates in a 96-well plate (Savinainen et al., 2014). The cellular background activity (both vector and AtABHD11 fraction) was negligible, and the enzyme activity was calculated after normalizing with the background activity. The phospholipase activity of AtABHD11 revealed that the maximum activity was observed with PS followed by PG and PE (Fig. 6B). The lysophospholipase activity was significantly higher with LPA followed by LPE and LPG (Fig. 6C). Further, a similar assay was validated with the available NBD-fluorescent fatty acid-labeled lyso(phospholipid) substrates, which confirmed the release of FFA from the corresponding substrates. Representative figure for fluorescent substrate assay for PS lipase activity was shown (Fig. 6B, inset). Acylhydrolase activity with nonpolar lipid substrates showed that the enzyme also had MAG lipase activity (Fig. 6D). Data are presented as the means of three independent experiments. Collectively, these experiments suggested that Arabidopsis ABHD11 is indeed an acylhydrolase with broader substrate specificity.

Altered Expression Profile of Lipid Genes in abhd11

The mutant and overexpression analyses confirmed the contribution of AtABHD11 to phospholipid metabolism. As shown in previous studies of the de novo pathway, excess phospholipids could be channeled to the synthesis of TAG. However, elevated levels of total phospholipid in the abhd11 mutant did not appear to influence the formation of TAG. Instead, total galactolipid levels were found to be elevated in the abhd11 mutant. We used the leaf microarray data of abhd11 mutant and wild-type tissues to assess the turnover of the excess phospholipid that accumulated in mutant leaves. As shown in the heat map (Fig. 7A; Supplemental Data S1), abhd11 mutant had 3080 and 2417 genes that were up- and down-regulated, respectively, by more than 2-fold compared with wild-type. Validation of the mRNA expression levels of lipid genes confirmed a more than 10-fold up-regulation of the MGD1, MGD2, MGD3, and DGD1 (approximately 8-fold) genes. Analysis of the genes involved in TAG biosynthesis showed that they were slightly up-regulated (Fig. 7B).

To validate the earlier experiment, AtABHD11 full-length gene was transiently overexpressed in both wild-type and abhd11 mutant plant leaves of 3 weeks old. Cauliflower mosaic virus 35s-driven AtABHD11 expression was confirmed by GUS assay. Untransformed wild-type and abhd11 leaves were used as control (Fig. 7C). Further, real-time PCR confirmed that both overexpressed and complemented leaves significantly lowered the transcript levels of MGD1, MGD2, MGD3, and DGD2, but DGD1 showed no significant effect as compared to vector control (Fig. 7D). In addition, polar lipid contents were analyzed in overexpressed

![Figure 8](image_url)

**Figure 8.** Schematic representation of the proposed role of AtABHD11. Model shows the role of Arabidopsis ABHD11 in both overexpressed and knock-down conditions. Dotted lines represent the limited lipid synthesis due to lipase activity of AtABHD11 when overexpressed. Red circles represent the genes that are up-regulated in the abhd11 mutant. G-3-P, Glycerol-3-phosphate; PAP, phosphatidic acid phosphatase; FAS, fatty acid synthase; Mgd1, monogalactosyldiacylglycerol synthase 1; Dgd1, digalactosyldiacylglycerol synthase 1.
vector and AtABHD11 leaf samples of the wild type and abhd11. The results indicated that the phospholipids (Fig. 7E) and galactolipids (Fig. 7F) were significantly lowered upon overexpression of AtABHD11. Together, these experiments revealed that AtABHD11 alters the expression of chloroplast lipid genes and polar lipid content in plant leaves.

**DISCUSSION**

ABHD-containing proteins have been shown to possess a wide variety of hydrolytic activities (Ollis et al., 1992). Recently, many studies have been carried out to understand the role of lipid molecules in plant growth and development. In plants, phospholipids are extensively hydrolyzed by phospholipases, which have been shown to be highly expressed during various stress conditions (Wang, 2001; Testerink and Munnik, 2005; Li et al., 2006; Wang et al., 2006). However, our understanding of the enzymes that are involved in lipid homeostasis remain insufficient. ABHD5, a human homolog in plants, has been characterized and shown to have LPA acyltransferase activity (Ghosh et al., 2009). Moreover, the disruption of ABHD5 in Arabidopsis caused the accumulation of nonpolar lipids in vegetative tissues (James et al., 2010).

In this study, we characterized AT4G10030 (AtABHD11) in both mutant and overexpression conditions. The abhd11 mutant seeds, when grown on soil, exhibited a higher rate of plant growth compared to the wild type. To date, characterization of ABHD proteins in different organisms revealed their direct contributions to various aspects of lipid metabolism (Ghosh et al., 2008a, 2008b, 2009; Long et al., 2011; Blankman et al., 2013). To assess whether AtABHD11 also exhibits a similar role, we analyzed the lipidomics data of Arabidopsis abhd11 mutant tissues. In 1995, Ohlrogge and Browse demonstrated that PC is the major phospholipid in Arabidopsis tissues. Interestingly, we observed a significant increase in the phospholipid content as PC > PG > PE > PI in abhd11 mutant tissues. Furthermore, quantitative analysis of leaf phospholipids molecular species revealed that the amount of unsaturated fatty acid containing lipids was significantly increased in the abhd11 mutant as compared to the wild type. An increase in phospholipids could occur because of either an increase in lysophospholipid acyltransferase activity or the reduced turnover of phospholipids.

To check for differences in the expression patterns of lipid biosynthetic genes in the abhd11 mutant, leaf whole-genome microarray profiles of both wild-type and abhd11 mutant tissues were compared. The gene expression profile did not show notable changes in the expression of genes that are involved in TAG biosynthesis. Interestingly, significant up-regulation was observed in chloroplast lipid biosynthetic genes. This observation also supported our quantitative lipid profiling in which the amount of TAG remains unchanged, while the galactolipid levels were found to be higher in abhd11 mutants. Based on the above observations, we hypothesized that phospholipids are possibly channeled toward galactolipid synthesis in leaves. An increase in the galactolipids of vegetative tissues in abhd11 mutants implicated their similarity with Atcgi58 mutants (James et al., 2010). However, the TAG levels were not significantly altered in abhd11 mutant plants, as reported in the cgi58 mutant. This finding clearly demonstrates the diversity of the catalytic activity of ABHD proteins. Quantitative real-time PCR analyses further confirmed our hypothesis that the mRNA levels were more than 10-fold higher for MGD1, -2, and -3 and 8-fold higher for DGD1, whereas the other lipid genes were up-regulated to a lesser extent.

It has been reported that the highest amount of 16:1 and 16:3 could be observed in chloroplast lipids in Arabidopsis (Ohlrogge and Browse, 1995). In accord with an earlier report, the galactolipid increase was observed with MGDG 34:6(18:3/16:3) and 36:6(18:3/18:3) and with DGDG 36:6(18:3/18:3) and 34:3(18:1/18:2). Moreover, there was also an increase in PC 36:5(18/3/18/2). Similar data were observed when a plastidial phosphatidic acid phosphatase (PAH) gene was mutated in Arabidopsis, resulting in higher amounts of PC due to the up-regulation of LPC acyltransferase (Wang et al., 2014). Unlike paah mutants, the abhd11 mutant did not cause marked changes in the expression of the LPCAT gene. There are two independent pathways that equally contribute to the synthesis of chloroplast lipids in Arabidopsis. The amount of PA was not altered in abhd11 mutants, and this could be because of the enhanced prokaryotic pathway in which PA serves as a precursor to synthesize MGDG. Increases in galactolipids also indicate the possibility of DAG as a precursor for the synthesis of various galactolipids. Overall, abhd11 mutant analysis suggests that its mutation caused an increase in phospholipids in Arabidopsis leaves; thus, these phospholipids were utilized efficiently by the MGD and DGD enzymes to generate more galactolipids in mutant leaves (Fig. 8).

The results obtained from mutant were verified by overexpression and complementation of the ABHD11 gene in its respective wild-type and abhd11 mutant plant leaves. Both gene expression and lipid analysis profiles correlated well with each other, which confirmed the involvement of AtABHD11 in polar lipid metabolism. To validate further, we hypothesized that ABHD11 is a possible phospholipase. Overexpression of AtABHD11 caused a significant reduction of phospholipids in E. coli cells. Interestingly, our in vitro experiments with individual substrates using purified recombinant AtABHD11 showed lipase activity toward phospholipids and lysophospholipids. However, a phospholipase assay using yeast total lipids as the substrate showed a slight variation regarding substrate preferences. When total lipid was used as a substrate, enzyme hydrolyzed PC more effectively than other lipids. This data also supports our earlier observation of PC increase in abhd11. These experiments suggested that the enzyme has broad substrate specificity and that the activity is dependent on the amount of substrate
available in the system. Additionally, minimum activity was observed with MAG as a substrate.

Our observation of \textit{abhd11} and the enhanced growth of mutant plants are in accord with earlier observations that the phospholipids act as plant growth regulators. It has been shown that exogenous application of phospholipids to plants induced hormone-like changes and led to cell expansion and delayed senescence (Chapman, 1998; Laxalt and Munnik, 2002; Meijer and Munnik, 2003). This study clearly confirms that AtABHD11 acts as a lyso(phospho)lipase and that the abolishment of the activity increases leaf phospholipids and DAG content, thereby increasing the galactolipid content in leaves of \textit{abhd11} in Arabidopsis.

MATERIALS AND METHODS

Materials

\[1^{\text{14}}\text{C} \text{Monoleoyl}-1\text{-rac-glycerol (MAG, 5 mCi/mmol)} \] was obtained from American Radiolabeled Chemicals. \[\text{[3]Acetate (51 mCi/mmol)} \] was obtained from Bhabha Atomic Research Centre (Mumbai, India). All of the fluorescent phospholipid substrates and lysolipids used in the enzyme assays and the lipid standards were from Avanti Polar Lipids. Oligonucleotide primers, anti-His-tag monoclonal antibody, and all other reagents were obtained from Sigma-Aldrich. Alkaline phosphatase substrate was from Perkin-Elmer. Restriction endonucleases and Pfu polymerase were from Thermo Scientific.

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In Silico Analysis

The primary sequence of the AtABHD11 protein was retrieved from The Arabidopsis Information Resource database. Homologs of AtABHD11 from Arabidopsis (\textit{Arabidopsis thaliana}) and other plant species were retrieved from the National Center for Biotechnology Information database; Sequences were subjected to construct a neighbor-joining tree. The plant AtABHD11 homologs were further analyzed for sequence similarity by multiple sequence alignment. Both analyses were carried out using CLC workbench software.

Plant Growth Conditions

\text{Arabidopsis Columbia-0 (wild-type) and abhd11 mutant lines were grown vertically on either soil or half-strength Murashige and Skoog medium supplemented with 0.5% (w/v) Suc and solidified with 0.8% agar. After 2 d of stratification at 4°C, seedlings were transferred to 25°C under a 16 h day (140 \text{mmol m}^{-2} \text{s}^{-1}) \text{and 8 h night regime.}

Characterization of \textit{abhd11} Mutant

T-DNA insertions in \textit{abhd11} mutant lines (salk_090222c, salk_109230, salk_090223, salk_090225, salk_090229, salk_109046, salk_090232, and salk_090227) were analyzed with a T-DNA left border and corresponding gene-specific primers as shown in Table I. A homozygous line was identified by PCR using genomic DNA as a template from wild-type and \textit{abhd11} mutant plants. The mutation was further confirmed by quantitative real-time PCR using cDNA synthesized from an equal amount of leaf total RNA. \textit{Actin} was employed as an internal control in both wild-type and \textit{abhd11} mutants.

Lipidomics Analysis of \textit{abhd11} Mutant and Wild-Type Plants

Total lipids were extracted from 100 mg of wild-type and salk_090222c (\textit{abhd11}) dried seeds or eight rosette leaves. Lipids were extracted and processed according to the described protocol (Welti et al., 2002; Wanjee et al., 2005). Lipids were analyzed at the KS Lipidomic Research Center by continuous infusion into an electrospray ionization source on a triple-quadrupole mass spectrometer (APLI4000, Applied Biosystems). The molecular species of lipids were quantified in comparison to the two internal standards using a correction curve determined between standards (Wanjee et al., 2005). Among five replicates, three replicates were chosen and used for further Student’s \textit{t test} to determine statistical significance. The values are represented as nmol of signals per extracted tissue dry weights. Phospholipid, MGDG, and DGDG amounts were represented as normalized mass spectral signal, which is normalized to the 1 nmol of two internal standards of that class (Vu et al., 2012).

Microarray Analysis

RNA isolation from \textit{abhd11} (salk_090222c) and wild-type Arabidopsis was performed using a Quagen RNA plate mini-kit following the manufacturer’s protocol with DNase treatment. The RNA concentration and purity were determined at an optical density ratio of 260/280 using a Nanodrop ND-1000 spectrophotometer, and the integrity of total RNA was verified on an Agilent 2100 Bioanalyzer using a RNA 6000 Nano LabChip.

The samples used for gene expression were labeled using an Agilent Quick- Amp labeling kit (p/n5190-0442). Total RNA (500 ng) was reverse transcribed at 40°C using an oligo(dT) primer tagged to a 17 mer polyromerase and converted to double-stranded cDNA. The synthesized double-stranded cDNA molecules were used as a template for cRNA generation. The cRNA was generated by in vitro transcription, and the dye Cy3 (Aptigent) was incorporated during this step. The cDNA synthesis and in vitro transcription steps were carried out at 40°C. Labeled cRNA was cleaned up using Quagen RNAse columns. The labeled cRNA (600 ng) sample was fragmented at 60°C, and hybridized onto genotypic designed custom Arabidopsis 8 \times 60K (AMADDID No: 037661) arrays. Fragmentation of the labeled cRNA and hybridization were carried using a gene expression hybridization kit (Agilent Technologies). Hybridization was carried out in Agilent’s SureHyb chambers at 65°C for 16 h. Hybridized slides were washed using Agilent gene expression wash buffers (Part No. 5188-5027) and scanned using an Agilent Microarray Scanner (Part No. G2600D).

Data extraction from images was carried out using Feature Extraction software Version 11.5 (Agilent). Raw data were analyzed using GeneSpring GX software from Agilent. Significant genes that were up- and down-regulated showing 1-fold (log base 2) and greater changes within samples with respect to control. Genes were classified based on the functional category and pathways using Genotypic Biointerpreter-Biological Analysis Software.

Quantitative Real-Time PCR

Wild-type and \textit{abhd11} total RNA were isolated from leaves using a Sigma Plant Total RNA isolation kit. Next, 1 µg of total RNA was used to prepare cDNA using a Thermo Scientific RevertAid H Minus first-strand cDNA synthesis kit. Primers were designed using Primer Express software 3.0 (Applied Biosystems), and the sequences are listed in Table I. The quality and quantity of the RNA were determined using Nanodrop. Further, amount of cDNA in both wild-type and \textit{abhd11} were confirmed with actin primers. Quantitative real-time PCR mix was prepared using cDNAs (1:20 dilution) with Power SYBR Green PCR Master Mix. The mRNA expression levels were analyzed in triplicate using an Applied Biosystems machine (SDS2.1). The quantitative real-time PCR data are presented as the means of three biological replicates, and the data were analyzed using Student’s \textit{t test}.

Transient Expression of \textit{AtABHD11}

Eight Arabidopsis leaves of 3 weeks old were infected with \textit{Agrobacterium tumefaciens} strain (GV3101) carrying the control cauliflower mosaic virus 35S promoter-driven PB121 vector or PB121-\textit{ABHD11} fusion construct. Prior to infection, bacterial cells carrying empty vector or the \textit{AtABHD11} construct were grown individually overnight in YEP medium containing rifampicin (50 \text{µg/mL}) and kanamycin (50 \text{µg/mL}). The cells were pelleted, washed, and resuspended in in infection medium (5.55 mM Glc, 60.25 mM K2HPO4, 33.04 mM KH2PO4, 10 mM Na2SO4, 2.34 mM sodium citrate, 7.57 mM (NH4)2SO4, 5.35 mM K2Cr2O7, 43.43 mM glycerol, 10 mM MES, pH 5.6, and 100 mM acetosyringone) and grown for 5 h at 30°C. The cells were pelleted and resuspended in infiltration medium (10 mM MgSO4 and 10 mM MES, pH 5.6, to an optical density of 0.5, followed by the addition of 200 µM acetosyringone). After 48 h of infiltration, leaves were used for the GUS assay (Ueki et al., 2008) to confirm the \textit{ABHD11} expression. Untransformed wild-type and \textit{abhd11} mutant leaves were used as controls for GUS assay. Real-time PCR was performed as mentioned earlier using cDNA prepared from equal amount of transiently overexpressed plant leaves, and the results were compared with the vector control. The overexpressed leaves were also harvested for lipid analysis as described earlier. The total lipids were run
Cloning and Expression of AtABHD11

Arabidopsis wild-type cDNA was used to amplify AtABHD11 using gene-specific primers as given in Table I. The gene was cloned into a pRSET-A vector using BamHI and KpnI restriction sites. A positive clone was confirmed by double-digestion, and the accuracy of the sequence was confirmed by DNA sequencing using T7 forward and reverse primers. The AtABHD11 gene construct was transformed into Escherichia coli BL21 (AID) cells. Cells were then induced with 0.5 mM isopropylthio-β-D-galactoside (IPTG) for 6 h at 37°C to enable gene expression. The cell pellets were resuspended in lysis buffer containing 50 mM Tris–HCl, pH 8.0, and 300 mM NaCl, 10% glycerol, 2 mM MgCl2, and 1 mM PMSF. Cells were disrupted by sonication and then the sample was subjected to centrifugation (9000 rpm) for 10 min followed by ultracentrifugation for 90 min (35000 rpm) at 4°C. AtABHD11 expression was confirmed by western-blot analysis using an anti-His monoclonal antibody (1:5000, v/v). Vector protein was used as a negative control. For protein purification, the supernatant containing His-tagged recombinant protein was allowed to bind to Ni2+-NTA agarose beads. After the collection of the flow-through, the column was washed with lysis buffer containing 20 mM imidazole. The bound protein was eluted with 250 mM imidazole in lysis buffer. The amount of purified recombinant protein was estimated using Lowry method (Hartee, 1972). Bovine serum albumin was used as a standard to calculate the amount of protein. The protein purity was analyzed by resolving onto 12% (w/v) SDS-PAGE followed by Coomassie Brilliant Blue staining. The purified recombinant protein was subjected to dialysis overnight at 4°C, and the enzymatic assay was then performed.

Determination of Fatty Acid Release

The acylhydrolase activity of AtABHD11 was calculated by estimation of released FFA using a fluorescent FFA estimation kit (Cayman Chemical, Cat. No. 700310; Savinainen et al., 2014). Briefly, the enzyme assay was performed by above-mentioned standard conditions, and the released FFA was coupled via three-step enzymatic reaction to produce H2O2-dependent generation of resorufin and the fluorescence monitored (Ex/em) using Thermo Scientific Varioskan Flash Multimode Reader. The amount of FFA was calculated by normalizing with respective controls, such as background, no enzyme control, and heat-inactivated controls.

Accession Number

The microarray data for wild-type and salk_090222c has been deposited in Genbank (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70672) and the accession number assigned as GSE70672.

Supplemental Data

The following supplemental materials are available.

Supplemental Data S1. Microarray analyses of abhd11 mutant and wild-type leaves.

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