2017

The 3-hydroxyacyl-ACP dehydratase component of the plant mitochondrial fatty acid synthase system

Xin Guan
_Iowa State University_

Yozo Okazaki
_RIKEN Center for Sustainable Resource Science, Yokohama, Japan_

Andrew Lithio
_Iowa State University, lithio@iastate.edu_

Ling Li
_Iowa State University, liling@iastate.edu_

Xuefeng Zhao
_Iowa State University, xzhao@iastate.edu_

Follow this and additional works at: http://lib.dr.iastate.edu/gdcb_las_pubs

Part of the Biochemistry, Biophysics, and Structural Biology Commons, Bioinformatics Commons, Genetics and Genomics Commons, and the Plant Breeding and Genetics Commons

The complete bibliographic information for this item can be found at http://lib.dr.iastate.edu/gdcb_las_pubs/160. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.
Authors
Xin Guan, Yozo Okazaki, Andrew Lithio, Ling Li, Xuefeng Zhao, Huanan Jin, Dan Nettleton, Kazuki Saito, and Basil J. Nikolau
Running Title: Mitochondrial fatty acid synthase

Discovery and characterization of the 3-hydroxyacyl-ACP dehydratase component of the plant mitochondrial fatty acid synthase system

Xin Guan,a,b,1 Yozo Okazaki,c Andrew Lithio,d Ling Li,e,2 Xuefeng Zhao,f,3 Huanan Jin,a,4 Dan Nettleton,d Kazuki Saito,c,g and Basil J Nikolaua,b,h,*

a Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011, USA; b The NSF Engineering Research Center for Biorenewable Chemicals (CBiRC), Iowa State University, Ames, Iowa 50011, USA; c Metabolomics Research Group, RIKEN Center for Sustainable Resource Science, Yokohama 230-0045, Japan; d Department of Statistics, Iowa State University, Ames, Iowa 50011, USA; e Department of Genetics, Development, and Cellular Biology, Iowa State University, Ames, Iowa 50011, USA; f Laurence H. Baker Center for Bioinformatics and Biological Statistics, Iowa State University, Ames, Iowa 50011, USA; g Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 260-8675, Japan; h Center for Metabolic Biology, Iowa State University, Ames, Iowa 50011, USA; 1 Current address: Department of Chemical Engineering, Stanford University, Stanford, CA 94305, USA; 2 Current address: Department of Biological Sciences, Mississippi State University, Starkville, Mississippi 39762, USA; 3 Current address: Information Technology, College of Liberal Arts and Sciences, Iowa State University, Ames, Iowa 50011, USA; 4 Current address: College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China

* For correspondence (e-mail dimmas@iastate.edu)

All author contributions
X.G and B.J.N. designed the research; Y.O. and K.S. analyzed the lipidome; L.L. and X.G. sequenced the transcriptome; X.Z. assembled the transcriptome; A.L. and D.N. performed statistical analyses of the transcriptomic data; X.G. and H.J. performed the in vitro enzymatic assays; X.G. and B.J.N. carried out all other experiments. X.G. and B.J.N. coordinated the preparation of the manuscript, and all authors contributed to the analysis of the collected data and writing of the manuscript.

Funding information
This work was partially supported by the National Science Foundation (Awards IOS1139489, EEC0813570 and MCB0820823 to B.J.N.); the Strategic International Collaborative Research Program of Japan Science and Technology Agency (Metabolomics for a Low Carbon Society, JST-NSF) (to K.S.); the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health and the joint National Science Foundation/NIGMS Mathematical Biology Program under award number R01GM109458 (to D.N.); and Iowa State University’s Plant Sciences Institute Scholars Program (D.N.).
We report the characterization of the Arabidopsis 3-hydroxyacyl-acyl carrier protein (ACP) dehydratase (mtHD) component of the mitochondrial fatty acid synthase (mtFAS) system, encoded by AT5G60335. The mitochondrial localization and catalytic capability of mtHD were demonstrated with a green fluorescent protein (GFP) transgenesis experiment, and by in vivo complementation and in vitro enzymatic assays. RNAi knockdown lines with reduced mtHD expression exhibit traits typically associated with mtFAS mutants, namely a miniaturized morphological appearance, reduced lipoylation of lipoylated proteins, and altered metabolomes consistent with the reduced catalytic activity of lipoylated enzymes. These alterations are reversed when mthd-rnai mutant plants are grown in a 1% CO₂ atmosphere, indicating the link between mtFAS and photorespiratory deficiency due to the reduced lipoylation of glycine decarboxylase. In vivo biochemical feeding experiments illustrate that sucrose and glycolate are the metabolic modulators that mediate the alterations in morphology and lipid accumulation. In addition, both mthd-rnai and mtkas mutants exhibit reduced accumulation of 3-hydroxytetradecanoic acid (i.e. a hallmark of lipid A-like molecules) and abnormal chloroplastic starch granules; these changes are not reversible by the 1% CO₂ atmosphere, demonstrating two novel mtFAS functions that are independent of photorespiration. Finally, RNA-Seq analysis revealed that mthd-rnai and mtkas mutants are near equivalent to each other in altering transcriptome, and these analyses further identified genes whose expression is affected by a functional mtFAS system, but independent of photorespiratory deficiency. These data demonstrate the non-redundant nature of the mtFAS system, which contributes unique lipid components needed to support plant cell structure and metabolism.

INTRODUCTION

Plant cells utilize at least three different fatty acid forming systems, which occur in multiple subcellular compartments: plastids, the membranes of the ER, and mitochondria (Ohlrogge and Jaworski, 1997, Samuels et al., 2008, Wada et al., 1997). Plastidial (ptFAS) and mitochondrial (mtFAS) fatty acid synthase systems form fatty acids de novo, whereas the ER-localized fatty acid elongase (FAE) system utilizes preexisting acyl-CoA precursors to synthesize very-long-chain fatty acids (VLCFAs) of 20-carbons and longer (James et al., 1995). The ptFAS system generates the bulk of a plant cell’s fatty acids from acetyl-CoA, and these fatty acids serve as precursors for the assembly of acyl-lipids that constitute membrane lipids (e.g. phospholipids and glycosylglycerolipids), storage lipids (e.g. triacylglycerol (TAG)) and signaling lipids (e.g. sphingolipids, phosphatidylinositols and oxylipins) (Li-Beisson et al., 2013, Benning, 2009). The VLCFAs generated by the FAE system can be incorporated into a variety of lipids, including surface cuticular lipids (Samuels et al., 2008), the ceramide moiety of sphingolipids (Markham et al., 2013), and in discreet quantities in some glycerolipids (Millar et al., 1998).
The mtFAS system appears to use free malonic acid as the substrate (Guan and Nikolau, 2016) to primarily generate octanoyl-acyl carrier protein (ACP), which is the required precursor for the biosynthesis of lipoic acid (Yasuno and Wada, 1998, Wada et al., 2001, Gueguen et al., 2000). Lipoic acid is the cofactor that is essential for pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (KGDH), branched-chain α-ketoacid dehydrogenase (BCKDH), and the glycine decarboxylase complex (GDC) (Taylor et al., 2004). To date, alternate functions for the mtFAS system have not been demonstrated, although its role in detoxifying mitochondrial malonic acid (Guan and Nikolau, 2016) and in remodeling cardiolipins (Frentzen and Griebau, 1994, Griebau and Frentzen, 1994) have been suggested.

Based on the characterization of three Arabidopsis mtFAS enzymatic components (i.e. mitochondrial β-ketoacyl-ACP synthase (mtKAS) (Yasuno et al., 2004, Olsen et al., 2004, Ewald et al., 2007), phosphopantetheinyl transferase (mtPPT) (Guan et al., 2015) and malonyl-CoA synthetase (mtMCS) (Guan and Nikolau, 2016)), it appears that the plant mtFAS system resembles the Type II FAS system that occurs in bacteria and plant plastids (White et al., 2005, Ohlrogge and Jaworski, 1997). Type II FAS systems recruit ACP as the carrier of the intermediates of the process and utilize dissociated, mono-functional enzymes to catalyze the iterative reactions that produce fatty acids (Hiltunen et al., 2010). This contrasts with the Type I FAS that occurs in the cytosol of fungi, mammals and some bacteria, where a multifunctional protein that contains all of the catalytic centers required for fatty acid biosynthesis iteratively catalyzes the formation of fatty acids from acetyl-CoA and malonyl-CoA (Smith et al., 2003).

Here, we report the identification and characterization of a gene encoding the mitochondrial 3-hydroxyacyl-ACP dehydratase (mtHD) that catalyzes the third of the four iterative reactions that constitute the mtFAS cycle. Systematic investigations (i.e. biochemical, morphological, metabolomic and transcriptomic analyses) confirm its role in mtFAS and the important role this process has in supporting photorespiration. Moreover, these characterizations identify additional novel mtFAS functions in supporting the assembly of lipid A-like molecules and an unexpected function in maintaining chloroplastic starch granule morphology.
RESULTS

Biochemical identification of AT5G60335 as the Arabidopsis mtHD component

Sequence-based identification of a candidate plant mtHD gene is somewhat complex because the two well characterized mtHD homologs from yeast (Kastaniotis et al., 2004) and humans (Autio et al., 2008b) share heterogeneous and low sequence similarity with many genes from Plantae. For example, BLAST analysis with the yeast HTD2 sequence failed to identify any significant Arabidopsis homolog (e-value > 3.9), whereas parallel analysis with the human HsHTD2 identified a single mtHD candidate AT5G60335 (e-value = 3e-17) (Figure 1).

The AT5G60335 protein coding sequence (CDS) was cloned with an RT-PCR strategy using an RNA template isolated from aerial organs of young Arabidopsis seedlings. This CDS encodes a protein of 166 amino acids, which contains an N-terminal 25 residue segment that is not homologous with human HsHTD. This N-terminal sequence is rich in basic amino acids and lacks acidic residues (Figure 1), these being characteristics typical of mitochondrial targeting presequence elements. The AT5G60335-encoded protein is predicted to be mitochondrially localized by MitoProt II (24 N-terminal residues with a score of 0.9957) (Claros and Vincens, 1996), PSORT (with a score of 0.751) (Nakai and Horton, 1999), and Target P (17 N-terminal residues with a score of 0.680) (Nielsen et al., 1997, Emanuelsson et al., 2000).

The AT5G60335 CDS was expressed in the yeast htd2 mutant strain that lacks a functional mtHD enzyme. This strain cannot grow on glycerol as sole carbon source due to a respiratory deficiency (Kastaniotis et al., 2004). In this experiment the putative mitochondrial targeting presequence (24 residues) of the AT5G60335 protein was replaced by the mitochondrial presequence of the yeast COQ3 protein (Hsu et al., 1996) to ensure the correct mitochondrial localization of the AT5G60335 protein in yeast.

As illustrated in Figure 2, neither the empty plasmid control nor the COQ3 mitochondrial targeting element are capable of rescuing the growth deficiency of the htd2 mutant strain on glycerol, but both the native yeast HTD2 gene and AT5G60335 CDS restored the growth of the yeast strain on glycerol media. This experiment therefore establishes that the AT5G60335 gene codes for a function that overcomes the deficiency in 3-hydroxyacyl-ACP dehydratase activity that is capable of generating acyl-ACP that is used to synthesize lipoic acid in yeast.

In vitro characterization of the enzymatic activity of the AT5G60335-coding protein

The E. coli produced recombinant AT5G60335-coding protein was assayed for the ability to catalyze the 3-hydroxyacyl-ACP dehydratase activity. As with 3-hydroxyacyl-ACP dehydratases characterized from a variety of different Type II FAS systems (Kastaniotis et al., 2004, Autio et al., 2008b, Autio et al., 2008a), this reaction was assayed in the reverse direction, namely the hydration of enoyl-CoA substrates. In addition, this assay was used to determine the substrate specificity of the enzyme in relation to the acyl
chain length of the substrate, and these data were compared to the specificity of mtKAS enzyme.

Two enoyl-CoA thioesters (i.e. trans-Δ²-10:1-CoA and trans-Δ²-16:1-CoA) were chemically synthesized as substrates for these assays (Supplemental Figure 1A), and
eight different acyl-ACP thioesters (with 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, and cis-Δ⁹-
16:1 acyl moieties) were synthesized as substrates for the mtKAS assays (Supplemental
Figure 1B). Both enzymes exhibited classical hyperbolic Michaelis-Menten activity
responses to increasing concentrations of each tested substrate, and $K_m$ and $V_{max}$ values
were calculated for each substrate-enzyme combination (Figure 3). The AT5G60335-
coding protein has the capability to catalyze the expected hydration of the enoyl-CoA thioesters, and catalytic efficiencies \((k_{cat}/K_m)\) for the medium-chain substrate \((\text{trans-Δ}^2-10:1\text{-CoA})\) is similar to the long-chain substrate \((\text{trans-Δ}^2-16:1\text{-CoA})\) (Figure 3A).

Similarly, mtKAS is able to use saturated acyl-ACP thioesters of between 4- and 16-carbon acyl chains as substrates, but its activity with the unsaturated substrate \((\text{cis-Δ}^9-16:1\text{-ACP})\) is too low for analysis.
16:1-ACP) was barely detectable (Figure 3B). The catalytic efficiency of mtKAS with different saturated acyl-ACP substrates (evaluated by $k_{cat}/K_m$) is ranked in the following orders: 16:0 > 14:0 > 6:0 > 8:0 > 10:0 > 12:0 > 4:0. This ranking is affected by differences in both $K_m$ and $V_{max}$ with each substrate. These characterizations indicate that plant mtFAS enzymes have the ability to synthesize saturated fatty acids of up to 18-carbon chain length.

**Mitochondrial localization of the AT5G60335 gene product**

The subcellular localization of the AT5G60335 protein was experimentally determined in Arabidopsis with a green fluorescent protein (GFP)-tagged transgenic fusion protein. In this experiment, the DNA fragment encoding the N-terminal 40-residues of the AT5G60335 protein was tested for its ability to target GFP to a specific organelle. Figure 4 shows confocal micrographs of roots and leaf mesophyll cells of the resulting GFP transgenic plants. In roots MitoTracker Orange was applied as the mitochondrial marker and it was simultaneously recorded with GFP fluorescence, while in mesophyll cells chlorophyll auto-fluorescence was used as a chloroplastic marker. In plants carrying the $p35S::AT5G60335$-GFP transgene, GFP signals were obtained from both roots and leaf mesophyll cells in a distinct pattern that indicates localization in organelles. This GFP feature overlaps with the MitoTracker Orange in roots, but is distinct from the leaf chlorophyll auto-fluorescence, revealing that the AT5G60335-GFP fusion protein is mitochondrially localized. In control experiments, no GFP fluorescence was detectable in roots and mesophyll cells of the wild-type plants; in transgenic plants carrying a non-targeted GFP construct (i.e. $p35S::GFP$), the GFP fluorescence localizes to the cytosol and nucleus, which is consistent with previous reports (Guan et al., 2015).

In combination therefore, these sets of experiments presented in Figures 1-4 lead us to the conclusion that AT5G60335 encodes the mitochondrial 3-hydroxyacyl-ACP dehydratase (mtHD) enzyme, and this organelle targeting process is guided by the N-terminal 40-residue leader sequence. Furthermore, the enzyme kinetic data reveal that in Arabidopsis, mtHD and mtKAS do not restrain the mtFAS system from synthesizing long-chain saturated fatty acids, of up to 18 carbons in length. This contrasts with other characterized eukaryotic mtFAS systems, where the substrate specificity of either mtHD (Autio et al., 2008a) or mtKAS (Zhang et al., 2005) constrain the system to produce fatty acids of 12 carbon atoms and shorter.

**Expression patterns of mtHD and mtKAS in different Arabidopsis organs**

The spatial and temporal expression pattern of the mtHD gene was determined by quantitative RT-PCR, and these data were directly compared to the other well-characterized mtFAS gene, mtKAS (AT2G04540). Quantitative RT-PCR analysis of RNA preparations extracted from different organs shows that the two mtFAS genes exhibit parallel expression patterns. The expression of both mtHD and mtKAS genes occur in all organs tested (Figure 5), with an approximately 3-fold difference between the highest (i.e. flowers) and lowest (i.e. siliques) levels of expression. These near ubiquitous expression patterns of the mtHD and mtKAS genes are consistent with the microarray data.
visualized by the Arabidopsis eFP Browser (Winter et al., 2007, Schmid et al., 2005), and are also comparable with the expression pattern of other already characterized mtFAS components, such as the mtPPT (Guan et al., 2015) and mtMCS (Guan and Nikolau, 2016) genes.
Morphological alterations associated with *mthd-rnai* and *mtkas* mutations

The physiological significance of the *mtHD* gene was investigated by studying segregants identified among a family of plants generated from an Arabidopsis genetic stock that carries a T-DNA-tagged mutant allele (*mthd-1*) in the heterozygous state.
These characterizations demonstrated that the T-DNA-tagged mutant is not a null allele, and there was no obvious phenotypic difference between wild-type plants and those that were homozygous for the \textit{mthd-1} allele (Supplemental Figure 2).

As an alternative therefore, we generated RNAi transgenic plants, which express a suppressor of the \textit{mtHD} gene, under the control of the 35S promoter. Seventy-eight independent RNAi lines were recovered, and quantitative RT-PCR analysis determined the levels of remaining \textit{mtHD} transcript in the aerial organs of young seedlings at 16 days after imbibition (DAI). Two RNAi lines that exhibit the lowest \textit{mtHD} expression levels (3\% and 4\% of the wild-type levels) were designated as \textit{mthd-rnai-1} and \textit{mthd-rnai-2} strains, respectively, and these were used in subsequent analyses. These two mutant strains exhibit aerial organs that are significantly reduced in size as compared to the wild-type plants, and their developmental appearance was classified according to the systematic system developed by Boyes et al. (Boyes et al., 2001). Thus, at 16 DAI these mutant plants develop to stage 1.06 (i.e. exhibiting 6 rosette leaves that are longer than 1 mm in length), whereas the wild-type plants develop to stage 1.07, within the same time-period (i.e. exhibiting 7 rosette leaves that are longer than 1 mm in length) (Figure 6A).

When these \textit{mthd-rnai} mutant plants were grown in an atmosphere containing 1\% CO$_2$ they grew near normally (Figure 6A). The phenotypic reversal in the elevated CO$_2$ atmosphere is typical of photorespiratory deficiency (Somerville and Ogren, 1979); the elevated CO$_2$ levels inhibiting the oxygenation reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase, and thus reducing the levels of 2-phosphoglycolate, the starter metabolite of photorespiration. Furthermore, this phenotype is also conferred by mutations in other mtFAS components (e.g. \textit{mtkas} (Ewald et al., 2007), \textit{mtppt-rnai} (Guan et al., 2015), and \textit{aae13} (Guan and Nikolau, 2016)).

To test any additive effect of mutations in the \textit{mtHD}- and \textit{mtKAS}-catalyzed reactions, we generated double mutant stocks by transforming the \textit{mtkas-2} mutant line with the \textit{p35S::mtHD-RNAi} transgene. We recovered 41 independent transformants, and selected two lines that display the lowest \textit{mtHD} expression levels (8\% and 11 \% of the wild-type level, respectively) for additional analysis. In ambient air the double mutant lines exhibit a stronger growth defect as compared to the \textit{mtkas-2} parental plants. In our experimental conditions, at 16 DAI the growth of these double mutants is arrested at stage 1.02 (i.e. exhibiting 2 rosette leaves), whereas the \textit{mtkas-2} mutant plants develop to stage 1.05 (i.e. exhibiting 5 rosette leaves) (Figure 6B). In addition, these double mutant plants do not progress any further in development and eventually die, which contrasts with the situation with the \textit{mtkas-2} mutant, which progresses to maturity, although at a slower rate than the wild-type plants, and ultimately set seeds. When grown in the 1\% CO$_2$ atmosphere, the growth of these double mutants is reversed to near wild-type appearance, and these plants develop to stage 1.06 by 16 DAI (Figure 6B).
Chloroplast alterations associated with *mthd-rnai* and *mtkas* mutations

More detailed insights into the growth phenotype of *mthd-rnai* and *mtkas* mutants were obtained by examining leaves of these mutants by light microscopy and by transmission electron microscopy. These observations illustrate the distinct differences in the leaf cell...
morphology and ultrastructure between the wild-type plants and the mutants (Figure 7A). Specifically, when these plants are grown in ambient air, mesophyll cells are enlarged in the mutants, making the leaves thicker than the wild-type plants. Despite the fact that these mutants lack a mitochondrial biochemical function, the ultrastructure of this organelle is unaffected. The most dramatic ultrastructural alterations in the mutants are...
associated with chloroplasts. Thylakoid membrane assembly is affected by the mutations, with the thylakoid structures being sparser and less extensive. Furthermore, starch granule ultrastructure appears to be drastically altered in the mutant lines, with many small granules present; this compares to the typical 2-5 disc shaped structures that occur in the wild-type plants.

Most of these alterations in the leaf cell morphology and ultrastructure of the mutants (i.e. cell size, and thylakoid membrane) are reversed when the mutant plants are grown in the 1% CO₂ atmosphere (Figure 7B). The exception to this morphological reversal is the ultrastructure of the starch granules, which maintain the abnormal morphology as observed when the plants were grown in ambient air.

**Altered protein lipoylation states associated with mthd-rnai and mtkas mutations**

Mitochondrial lipoic acid biosynthesis is primed with octanoyl-ACP generated by the mtFAS system (Ewald et al., 2007). We therefore examined the protein lipoylation states of different enzymes in the mtFAS mutants (i.e. mthd-rnai, mtkas-2, and mtkas-2-mthd-rnai mutants). Using a western blot procedure with anti-lipoic acid antibodies we examined the lipoylation states of H subunit of GDC, E2 subunits of mitochondrial PDH and KGDH and plastidial PDH (Ewald et al., 2007, Guan et al., 2015) (Figure 8). Plants were grown in the 1% CO₂ atmosphere to eliminate any bias due to the morphological appearance associated with the mutant alleles. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein extracts does not indicate any dramatic differences in the expressed proteomes of these mutant plants. The most dramatic alteration in the lipoylation states of these proteins was associated with H subunit of GDC, which is depleted to about 10% of wild-type levels in the mthd-rnai mutants, and is undetectable in the mtkas-2 mutant, and mtkas-2-mthd-rnai double mutant strains. The dramatic depletion in the lipoylation status of this protein does not affect the accumulation of the H protein itself, as indicated by the measurements made using anti-H protein antibodies. Therefore, these plants harbor a large pool of inactive apo-H subunit of GDC.

These immuno-based lipoic acid analyses also identified that the lipoylation status of other mitochondrial lipoylated proteins is also reduced in these mutant plants; these are decreased to as low as 30% of the wild-type level (i.e. E2 subunit of KGDH in the mtkas-2-mthd-rnai double mutants). In all of these mutants, the lipoylation status of the E2 subunit of plastidial PDH was unaffected.

**Metabolomic alterations associated with mthd-rnai and mtkas mutations**

Using multiple analytical platforms, the metabolomes of the mthd-rnai and mtkas mutants were analyzed and compared to wild-type plants. These analyses quantified 143 metabolites in aerial organs, and these metabolites were categorized as aqueous metabolites, fatty acids, lipids, surface cuticular lipids, and starch granule components. The metabolomes of these mutants and wild-type plants were determined when they were grown in either ambient air, when the growth phenotype associated with the
photorespiratory deficiency is expressed, or grown in the 1% CO₂ atmosphere, when the photorespiratory growth phenotype is suppressed (Figure 9 and Supplemental Figure 3). In ambient air, the most dramatic metabolic alteration in aqueous metabolites is a 70- to 150-fold hyperaccumulation of glycine. Several additional aqueous metabolites exhibit
significant, but smaller increases in accumulation, of between 25% and 4-fold of the wild-type levels. In contrast, sucrose is depleted to between 5% and 12% of the level present in the wild-type plants.
All of these mutant plants exhibit significantly reduced levels of saponifiable fatty acids (e.g. 16:1, 16:2, and 16:3) that constitute membrane lipids. Consistent with the reduction in the accumulation of these fatty acids, the levels of the major leaf glycolipids (i.e. monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG)) are markedly reduced to below 80% of the wild-type levels. Chlorophyll content is also reduced to below 80% of the wild-type level, as would be expected from the yellowish appearance of the mutant plants. In addition, the accumulation of most surface cuticular lipids is significantly reduced in these mutants. In contrast however, TAG hyperaccumulates to above 5-fold of the wild-type level.

Alterations in the accumulation of phospholipids (i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI)) and diacylglycerol (DAG) are barely detectable. In addition, the quantities of insoluble starch and water-soluble glucose polysaccharide (WSP) were compared between the wild-type and mutant plants. Despite the difference in starch granule morphology observed by transmission electron microscopy, the total quantity of starch and WSP was essentially unchanged in the mutants.

These alterations in the metabolome appear to be a consequence of the deficiency in photorespiration, because most of these are reversed when the mutant plants are grown in a condition that inhibits photorespiration (i.e. the 1% CO2 atmosphere). An exception is glycine, which although is significantly reduced when these mutant plants are grown in the 1% CO2 atmosphere, it is still 15- to 50-fold higher than that in the wild-type plants.

We also compared the aqueous metabolite profiles between the mtkas-2-mthd-rnai double mutant and their parental mtkas-2 mutant plants. Whereas malonate levels are unaffected in the mtkas-2 or mthd-rnai single mutants (Figure 9), it is elevated by 3-fold in the double mutant (Supplemental Figure 4), even when these plants are grown in the 1% CO2 atmosphere, which suppresses photorespiration. This elevation in malonate levels, and associated growth penalty resembles the situation in the aae13-1 mutant (Guan and Nikolau, 2016), which cannot activate malonate to malonyl-CoA. Therefore, the reduced size of the mtkas-2-mthd-rnai double mutants maybe associated with malonate toxicity (Guan and Nikolau, 2016), a known inhibitor of succinate dehydrogenase activity (Quastel and Wooldridge, 1928, Greene and Greenamyre, 1995).

Biochemical Mimics of the mutant effect on the morphology and metabolome

Based on the observation that growing the mthd-rnai and mtkas mutant plants in an elevated CO2 atmosphere suppresses the changes in morphology and metabolome, we considered that some of these genetic deficiency effects are secondary to the deficiency in photorespiration. We hypothesized for example that the alterations in morphology and lipids are a result of the alterations in the steady state levels of soluble metabolites that are intermediates of photorespiration. This postulate was tested by exogenously feeding these photorespiration intermediates to the wild-type and mtkas mutant plants, and testing whether these biochemicals complement or mimic the mtkas mutation. Specifically, we targeted the effects of...
treating plants with sucrose, glycolate, and glycine; the metabolites associated with
photorespiration, whose accumulation is either down- or up-regulated by the
mutations.

By growing the wild-type and mtkas-2 mutant plants on media supplemented with 3%
sucrose, we tested whether the mtkas-induced reduction in sucrose accumulation
was the cause of the alterations in morphology and lipids. First, as indicated by
directly assaying the sucrose content of the tissue, we showed that these plants
take up the exogenously supplied sucrose (Supplemental Figure 5C), and the
sucrose depletion that occurs in the mtkas mutant plants is significantly alleviated,
reaching about 30% of the level obtained in the sucrose-treated wild-type plants.
The sucrose-supplementation markedly relieves the dwarf phenotype and the
yellowish leaf color of the mtkas mutant plants (Supplemental Figure 5A). In
addition, externally added sucrose reverses the mtkas-induced alteration in the
ultrastructure of the thylakoid membrane system (Supplemental Figure 5B).
Moreover, the sucrose treatment effectively complements the alterations in the
profiles of fatty acids, glycoglycerolipids and chlorophylls (Supplemental Figure 5C).
In contrast, exogenous sucrose does not reverse the enlarged mesophyll cells,
abnormal starch grains and hyperaccumulation of TAG in the mtkas-2 mutant.
These results demonstrate therefore that sucrose depletion in the mtkas-2 mutant
contributes to the morphological phenotype (i.e. stunted growth, defective thylakoid
membrane system and loss of chlorophylls) and the alterations in
glycoglycerolipids.

In corollary experiments we evaluated the effect of other exogenous biochemicals
(specifically 10 mM glycolate and glycine) on the wild-type plants to test whether
the mtkas-induced hyper-accumulation of these photorespiratory intermediates are
the cause of the morphological phenotype and of the altered lipidome in the
mutants. These exogenously provided biochemicals appear to have been taken up
by the plants as evidenced by the 4-fold increase in glycolate tissue levels in the
glycolate-treated plants (Supplemental Figure 5C); these elevated levels are
similar to those measured in the mthd-rnai and mtkas mutant plants. The
application of exogenous glycolate leads to a dramatic dwarf appearance with
dark-green leaf color (Supplemental Figure 5A). In addition, this treatment
increases the number of large starch granules in the chloroplasts (Supplemental
Figure 5B). In parallel, these plants also exhibit about a 6-fold increase in TAG
accumulation (Supplemental Figure 5C). These metabolic changes resemble those
observed in the mthd-rnai and mtkas mutant plants, which also hyperaccumulate
glycolate. In contrast, glycolate does not affect the accumulation of other portions
of the tested plant lipidome in a pattern that resembles the mthd-rnai and mtkas
mutants.

The contribution of glycine to the alterations in morphology and lipidome is somewhat
more difficult to adjudicate, as the exogenous application of glycine to the wild-type
plants only leads to an increase of the endogenous glycine levels by about 5-fold, which
is considerably lower than the over 70-fold hyperaccumulation that occurs in the mthd-rnai and mtkas mutants. Moreover, when the mtkas mutant phenotype is suppressed by growth in the 1% CO₂ atmosphere, endogenous glycine levels are still 15-fold higher than those that occur in the wild-type plants. It appears therefore that glycine hyperaccumulation is only a small contributor to those alterations in morphology and lipidome.

Depleted 3-hydroxytetradecanoic acid associated with mthd-rnai and mtkas mutations

In addition to the metabolic alterations discussed above, we also identified two novel fatty acids in Arabidopsis, namely 3-hydroxytetradecanoic acid and 3-hydroxyhexadecanoic acid (Supplemental Figure 6A). These are components that have long been known to be associated with Gram-negative bacterial lipid A (Raetz et al., 2007), and recently described with Arabidopsis lipid A-like molecules (Li et al., 2011). While the accumulation of 3-hydroxyhexadecanoic acid is unaffected in the mthd-rnai and mtkas mutant plants, the accumulation of 3-hydroxytetradecanoic acid is depleted to between 20% and 30% of the wild-type level, irrespective of the atmospheric conditions that affect photorespiration (Figure 9 and Supplemental Figure 3).

To clarify the metabolic origin of 3-hydroxytetradecanoic acid, we assayed its accumulation in two mutant strains that are deficient either in photorespiration (i.e. the shmt1-2 mutant that is deficient in the photorespiratory glycine to serine conversion (Voll et al., 2006)) or in the biosynthesis of lipid A-like molecules (i.e. the atlpxa-1 mutant that is deficient in the first reaction of the assembly of lipid A-like molecules (Li et al., 2011)) (Supplemental Figure 6B). 3-Hydroxyhexadecanoic acid content is unaffected in the shmt1-2 mutant, demonstrating that its depletion is independent of the photorespiratory deficiency. In the atlpxa-1 mutant however, the accumulation of 3-hydroxytetradecanoic acid is depleted to 15% of the wild-type level, indicating that lipid A-like molecules are the major metabolic sink of this hydroxylated fatty acid. The latter conclusion is further supported by the substrate specificity of acyl transferases (i.e. AtLpxA, AtLpxD1 and AtLpxD2) that are involved in the assembly of lipid A-like molecules; all of these enzymes recognize 3-hydroxytetradecanoyl-ACP as their optimal substrate (Joo et al., 2012) (Supplemental Figure 6C).

Parallel alterations in gene expression induced by the mthd-rnai-1 and mtkas-2 mutations

RNA-Seq experiments were performed to assess alterations in the transcriptomes associated with two independent genetic blocks in the mtFAS system, namely mthd-rnai-1 and mtkas-2 mutant strains. In these experiments we sequenced the transcriptomes of the wild-type and the two mutant strains that were grown in either ambient air or in the 1% CO₂ atmosphere. Initially we directly measured changes in gene expression by conducting quantitative RT-PCR on 9 target genes and tested the validity of using the count numbers obtained from the identical RNA sample preparations by RNA-Seq analysis. Correlation coefficients of the data obtained by the two methods are above 0.9
(Supplemental Figure 7), indicating that RNA-Seq read counts can be used as a quantitative readout of global changes in gene expression in response to the two mutations.

Statistical analyses investigated whether mthd-rai-1 and mtkas-2 elicit similar changes in the transcriptome. These analyses identified differentially expressed genes (DEGs) among the three genotypes (i.e. wild-type, mthd-rai-1 and mtkas-2), when these plants were grown in the two environmental conditions (i.e. ambient air or the 1% CO₂ atmosphere); these are comparisons 1 to 6 of Figure 10A (Supplemental Data 1). Gene Ontology (GO) functional categories associated with these DEGs were analyzed at TAIR (www.arabidopsis.org; Supplemental Data 2).

Comparisons 1 (7,626 DEGs; 37% of the expressed genome) and 2 (9,052 DEGs; 44% of the expressed genome) indicate that each mutation significantly alters expression of a large proportion of the genome when plants are grown in ambient air. A chi-squared test for association between the two DEG lists indicates a significant (p-value < 0.0001) overlap between genes affected by the mthd-rai-1 and mtkas-2 mutations. Furthermore, Figure 10B shows the plot of fold-change in expression in Comparison 1 versus Comparison 2 and indicates that the directions and magnitudes of the estimated effects of each mutation were strongly positively correlated (r = 0.943, p-value < 0.0001). However, Comparison 3 demonstrates that there are a few genes (114 DEGs; < 1% of the expressed genome) whose expression is differentially affected between the two mutants.

While the distinctions in gene expression profiles between the wild-type and mutants are greatly reduced when these plants were grown in the 1% CO₂ atmosphere, similar relationships between these three genotypes are apparent. Specifically, Comparisons 4 (286 DEGs; 1% of the expressed genome) and 5 (1,825 DEGs; 9% of the expressed genome) show strong correlated changes in the transcriptome relative to the differences between the two mutants, as reflected by Comparison 6 (63 DEGs; < 1% of the expressed genome). Again, a chi-squared test for association between the DEG lists for Comparisons 4 and 5 indicate significant overlap between the genes affected by the two mutations, and Figure 10B shows a positive correlation (r = 0.864, p-value < 0.0001) between the effects of each mutation. Collectively, these results demonstrate that the mthd-rai-1 and mtkas-2 mutations induce parallel changes in the transcriptome, which supports the hypothesis that these two genes operate in the same metabolic pathway, namely mtFAS.

**Transcriptomic alterations associated with mtFAS deficiency but independent of photorespiratory deficiency**

Further statistical analyses addressed whether any photorespiration-independent, but mtFAS-dependent processes are revealed by the RNA-Seq analysis of the mthd-rai-1 and mtkas-2 mutants. For these analyses we initially identified DEGs whose expression is associated with 3 modifiers of gene expression: 1) different genotypes (G-effect; i.e. wild-type, mthd-rai-1 and mtkas-2); 2) different environmental conditions (E-effect;
ambient air and the 1% CO₂ atmosphere); and 3) the interaction between genotypes and environmental conditions (i.e. GxE-effect). These analyses identified 13,514 DEGs (66% of the expressed genome), whose expression is associated with one or more of these three modifiers. Approximately half of these DEGs are singularly affected by the G-, E- or GxE-effects (Figure 11A; Supplemental Data 3 online for details). Multiple modifiers
combine to affect the expression of the remaining DEGs; these being combinations of 3 subsets that combine two modifiers (G and E, 15% of the DEGs); G and GxE (7% of DEGs); and E and GxE (8% of DEGs)), and one subset that combine all three modifies (17% of DEGs) (Figure 11A; Supplemental Data 3 online for details).
Additional insights on the metabolic impact of these modifiers was obtained by dissecting each of the 7 subsets illustrated by the Venn diagram shown in Figure 11A into clusters that visualize the expression pattern relative to the three genotypes and 2 different environmental conditions (Figure 11B). For example, the 1390 DEGs that respond only to the G-effect can be separated into two clusters: Cluster 1 contains DEGs that are up-regulated in each mutant, and Cluster 2 contains DEGs that are down-regulated in each mutant, but all of these genes are minimally affected by the change in environmental conditions. These are therefore genes whose expression is affected by the mtFAS deficiency, irrespective of the atmospheric CO2 concentration. Therefore, these DEGs are probably not associated with the photorespiratory deficiency, as the latter would be expected to show distinct gene expression patterns between plants grown in ambient air and the 1% CO2 atmosphere.

Similarly, DEGs that respond only to the E-effect can be separated into two clusters: Cluster 3 contains DEGs whose expression is higher in the 1% CO2 atmosphere, whereas Cluster 4 contains DEGs whose expression is lower in the 1% CO2 atmosphere, but all of these genes are minimally affected by differences in genotypes (Figure 11B). These DEGs are therefore responding mainly to the change in the atmospheric CO2 concentration, and not to the mtFAS deficiency.

DEGs that respond to only the GxE-effect can also be separated into two clusters. But these DEGs demonstrate interdependence between G- and E-effects in altering gene expression (Figure 11B). Specifically, Cluster 5 contains DEGs whose expression is up-regulated in each of the mutants when grown in ambient air, but they are down-regulated when grown in the 1% CO2 atmosphere. DEGs in Cluster 6 are oppositely affected, in that expression of these genes is down-regulated in the mutants in ambient air, but up-regulated in the mutants in the 1% CO2 atmosphere.

The subsets that integrate two or more modifiers of gene expression are clustered in multiple expression patterns, and these appear to be additive of the patterns of each individual modifier (Figure 11B). For example, genes whose expression is affected by both G-effect and E-effect can be separated into 6 clusters (Clusters 7 to 12). Similarly, Clusters 13 to 34 are identifiable by examining the patterns of gene expression that respond to two (i.e. G and GxE; E and GxE effects) or all three modifiers. The expression of these latter DEGs show complex patterns that are associated with both the mtFAS deficiency and variations in the atmospheric CO2 concentration. An example is cluster 14, which contains DEGs whose response to genotypes is restricted to only the ambient air condition. Because the altered expression of DEGs in this cluster is suppressed when plants are grown in the 1% CO2 atmosphere, we associate these genes as responding to the mtFAS-dependent photorespiratory deficiency.

By generally applying this rationale we were able to identify clusters as containing DEGs that are primarily associated with the mtFAS deficiency, and distinguish them from those that are associated with the change in the atmospheric CO2 concentration, with the photorespiratory deficiency, or combination thereof (Figure 11C).
Biological functionalities of DEGs associated with the mtFAS-dependent processes, but independent of photorespiratory deficiency were explored by GO enrichment analyses using AmiGO (Carbon et al., 2009). These analyses focused on gene annotations associated with DEGs in expression Clusters 1, 2, and 7-12. Supplemental 8A visualizes the results in the form of heat maps that categorize the DEG annotations with GO biological processes, GO subcellular components and GO molecular functions (See Supplemental Data 4 online for details). These analyses indicate that these 8 expression clusters separate into three distinct clades, namely the clade that contains Cluster 1, the clade that contains Cluster 10, and the clade that contains Clusters 2, 7, 8, 9, 11 and 12. Despite the fact that the 3 clades contain DEGs whose expression patterns are distinct, the GO annotation terms that are enriched with each clade are quite similar. This similarity is visualized in Tag Clouds that identifies words enriched in the GO annotation terms (Supplemental 8B). Thus, all three clades have GO annotation terms that are rich in a few key words. From the Biological Process category, these words are “biosynthetic and metabolic processes”; from the Subcellular Compartment category, these words are “intracellular, organelle, complex and membrane”; and from the Molecular Function category, these words are “binding, activity, enzyme classes such as hydrolase and ligase, and metabolites such as purines, nucleosides and nucleotides”. The enrichment in these terms appears to indicate that mtFAS-dependent processes that are independent of photorespiration, maybe associated with the metabolism of nucleotides and nucleosides, and particularly associated with chloroplasts or plastids. This latter term-enrichment is consistent with the data presented in Figure 7, which demonstrated that the most dramatic ultrastructural alteration in these mtFAS mutants are associated with alterations in the morphology of the chloroplasts and thylakoid membrane assembly.
DISCUSSION

The occurrence of a FAS system in mitochondria was first implied by the discovery of a mitochondrial located ACP in Neurospora (Zensen et al., 1992). Since then, this FAS system has been characterized in yeast and humans (Hiltunen et al., 2010), confirming its multi-component, Type II character. In plant cells, to date only the mtACP isoforms (Shintani and Ohlrogge, 1994, Meyer et al., 2007), mtKAS (Yasuno et al., 2004, Olsen et al., 2004, Ewald et al., 2007), mtPPT (Guan et al., 2015) and mtMCS (Guan and Nikolau, 2016) have been experimentally identified and characterized as components that support mitochondrial fatty acid biosynthesis. In this study we identified and characterized the Arabidopsis genetic locus AT5G60335 as encoding the mtHD component of the Arabidopsis mtFAS system, which catalyzes the third iterative reaction of the FAS cycle, the dehydration of 3-hydroxyacyl-ACP intermediate. Evidence that supports this conclusion includes: 1) the mitochondrial localization of the protein encoded by the AT5G60335 gene locus, demonstrated by the use of a transgenic GFP gene-fusion; 2) the genetic complementation of the growth deficiency of the htd2 yeast mutant strain that lacks mtHD catalytic function; 3) the in vitro characterization of the recombinant Arabidopsis AT5G60335-encoded protein, which directly demonstrates that this protein catalyzes the reverse reaction, the hydration of enoyl-CoA substrates; and 4) the demonstration that mutant strains deficient in the AT5G60335 gene and those deficient in the mtKAS gene (a known catalytic component of mtFAS) exhibit near identical phenotypic, metabolomic and transcriptomic responses.

mtFAS-dependent functionalities were further investigated by a combined global transcriptomic and metabolomic analyses of mutants deficient in mtHD (i.e. mthd-rai mutants) and mtKAS (i.e. mtkas mutants), each of which reduces mtFAS activity. These analyses expanded the understanding of the central role of mtFAS in lipoic acid homeostasis, which is of major significance in supporting photorespiration via the lipoylation of the H subunit of GDC. In addition, these analyses established novel functionalities associated with mtFAS; these being the generator of the fatty acid components for the assembly of lipid A-like molecules, and via an as yet unknown mechanism affecting the morphology of chloroplastic starch granules.

mtHD contributes to photorespiration

Previous characterizations of the Arabidopsis mtFAS components (i.e. mtKAS (Yasuno et al., 2004, Olsen et al., 2004, Ewald et al., 2007), mtPPT (Guan et al., 2015) and mtMCS (Guan and Nikolau, 2016)) have implicated a role for mtFAS in supplying octanoyl-ACP as the precursor to synthesize mitochondrial lipoic acid, which is an essential cofactor of the H subunit of GDC, a crucial component of photorespiration. The mthd-rai mutants characterized herein exhibit metabolic alterations that are also primarily associated with this inability to maintain photorespiration. Specifically, photorespiratory glycine hyperaccumulates, and sucrose and fatty acid accumulation is decreased; these metabolic alterations are reversed when these mutant plants are grown in a condition that suppresses photorespiration (i.e. a 1% CO₂ atmosphere).
Therefore, the role of mtFAS to support lipoic acid biosynthesis appears to be near universal among all clades of eukaryotic organisms, as evidenced by characterization of the yeast, human, and plants (Hiltunen et al., 2010, Wada et al., 1997).

To expand on the previously identified traits associated with mtFAS metabolism, we conducted a more comprehensive metabolomic analysis of the lipid species of *mthd-rnai* and *mtkas* mutants. Analyses of the lipidome and cuticle of these mutants reveal alterations in several lipid classes, including reduced accumulation of glycerolipids, chlorophylls and surface cuticular lipids, and increased accumulation of leaf TAG. Somewhat unexpectedly however, these alterations in lipid metabolism are associated with non-mitochondrial compartments (i.e. chloroplasts). These findings are evidenced by the ultrastructural phenotype of chloroplasts, namely less extensive thylakoid membranes that are composed of glycerolipids and chlorophylls. Given the fact that these alterations in lipids and chloroplastic ultrastructure are completely reversed when these plants are grown in a non-photorespiratory condition (i.e. 1% CO2 atmosphere), they are likely to be secondary to the photorespiratory deficiency.

Some of these metabolomic changes can be interpreted with the global transcriptomic data revealed by the RNA-Seq analysis of the two mtFAS mutants, for example surface cuticular lipid biosynthesis and plastidial TAG biosynthesis. In the former case, a few genes involved in the surface lipid biosynthesis (e.g. CER1, CER3 and CER5 (Samuels et al., 2008)) exhibit reduced expression levels in ambient air, which is reversed by the 1% CO2 treatment. Considering the essential catalytic roles of CER1 and CER3 in producing alkanes from very-long-chain fatty acids (Bernard et al., 2012), and the role of the CER5 (i.e. an ABC transporter) in exporting surface cuticular lipids across the plasma membrane (Pighin et al., 2004), the reduced accumulation of surface cuticular lipids in the mtFAS mutant plants is consistent with these transcriptional alterations. In the latter case, the DGAT1 gene exhibited increased expression levels in ambient air, and this was reversed by the 1% CO2 treatment. This finding reflects the importance of DGAT1 as a major regulator of the TAG biosynthetic pathway in seedling plants, as evidenced by the previous analysis of *dgat1* mutants, which display a major reduction in leaf TAG accumulation (Zhang et al., 2009, Tjellstrom et al., 2015).

These examples illustrate the complexity of integrating transcriptomic and metabolomic data, which is also implied by principles of Metabolic Control Analysis (MCA) (Fell, 1992). Specifically, the regulation of surface cuticular lipid biosynthesis and plastidial TAG biosynthesis exemplify a regulatory network that focuses the regulation of a metabolic pathway at a few reactions within a pathway (e.g. CER1-, CER3-, and DGAT1-catalyzed reactions).

Other complexities that can contribute to weak correlations between transcriptomic and metabolomic data are the role of post-transcriptional (Gygi et al., 1999, Tian et al., 2004) and post-translational regulatory mechanisms. Particularly, post-translational regulation was exemplified in this study by the depletion of the essential lipoylation of H protein of...
GDC that inactivates the catalytic activity of this key enzyme of photorespiration, although the expression of H protein is normal in the mutant strains.

**mtFAS contributes fatty acid component for the assembly of lipid A-like molecules**

It is generally believed that the substrate specificities of the mtHD and mtKAS enzymes are the major factors that determine the chain lengths of fatty acids that mtFAS systems can produce. For example, acyl-substrates that are longer than 12 carbon atoms in length are poor substrates for the mtHD component in *Trypanosoma brucei* (Autio et al., 2008a) and the mtKAS component in humans (Zhang et al., 2005), which limits the fatty acid products of the mtFAS systems in these organisms. In contrast, our *in vitro* characterizations of the recombinant mtHD and mtKAS proteins demonstrate that these two enzymes display broader substrate specificity with respect to the chain lengths of substrates, up to 16 carbon atoms. Thus, we conclude that in Arabidopsis, mtHD and mtKAS may not necessarily limit the chain lengths of the fatty acids that are generated by the plant mtFAS system. The Arabidopsis mtFAS system therefore may be able to produce both octanoyl-ACP for lipoic acid biosynthesis and longer-chain acyl-ACP of potentially up to 18 carbons in length.

Consistent with this finding, we discovered that Arabidopsis cells appear to source 3-hydroxytetradecanoate from the mtFAS system to assemble lipid A-like molecules. This is evidenced by the fact that the accumulation of this fatty acid is depleted in the *mthd- rnai* and *mtkas* mutants, and its accumulation is also depleted in a mutant deficient in the assembly of lipid A-like molecules (i.e. *atlpxa-1*), but is unaffected in a photorespiratory mutant (i.e. *shmt1-2*), irrespective of atmospheric CO₂ conditions. In addition, the acyl transferases (i.e. AtLpxA, AtLpxD1 and AtLpxD2) that can use 3-hydroxytetradecanoyl-ACP as the substrate for assembly of lipid A-like molecules are mitochondrially located (Seveno et al., 2010, Li et al., 2011). Considering the proteobacterial origin of mitochondria (Timmis et al., 2004), the finding that mtFAS generates the acyl-moieties of lipid A-like molecules may imply that both the mtFAS system and the synthetic machinery for assembling lipid A-like molecules were acquired from the ancient proteobacterium that evolved to give rise to the modern mitochondria.

The Arabidopsis mtFAS system therefore, not only generates the octanoyl-ACP precursor for lipoic acid biosynthesis (Ewald et al., 2007, Guan et al., 2015, Guan and Nikolau, 2016), but also the 3-hydroxytetradecanoyl-ACP that is the precursor for the assembly of lipid A-like molecules. It’s interesting to note that the mtFAS system appears to terminate the fatty acid elongation cycle via acyl transferase mechanisms, which is distinct from the ptFAS system that terminates the elongation cycle via a hydrolytic mechanism, catalyzed by acyl-ACP thioesterases (Jing et al., 2011). Specifically, mtFAS uses the acyl-transferases AtLpxA, AtLpxD1 and AtLpxD2 (Li et al., 2011) for the assembly of lipid A-like molecules, and a combination of lipoyl transferase (Wada et al., 2001) and lipoic acid synthase (Yasuno and Wada, 1998) to generate lipoic acid.
The physiological functions of plant lipid A-like molecules are unclear (Armstrong et al., 2006). Because no morphological differences have been observed in plants that carry null alleles of genes that synthesize these molecules (Seveno et al., 2010, Li et al., 2011), it appears that lipid A-like molecules are not crucial for normal plant growth and development. Another unknown in this area of plant metabolism is the question of whether the four characterized lipid A-like molecules (i.e. lipid X, UDP-2,3-diacyl-GlcN, Disaccharide-1-P and lipid IVβ (Li et al., 2011)), which are metabolic intermediates in the assembly of lipid A in Gram-negative bacteria, are intermediates of metabolism in plants, or they represent the final products of the plant biosynthetic pathway. If they are intermediates, then what is the chemical nature of the final product that is produced by plants from these intermediates? Regardless, the fact that genes of biosynthesis of lipid A-like molecules have been maintained during the evolution of plants, post the endosymbiotic events that evolutionarily gave rise to mitochondria, may be indicative of an unknown important physiological functions that confer an evolutionary advantage.

Starch granule morphology is dependent on a functional mtFAS system

An abnormal starch granule morphology occurs in the mthd-rnai and mtkas mutants, and this is maintained irrespective of an atmospheric growth condition that suppresses photorespiration. Therefore, starch granule morphology is a trait that is dependent on mtFAS function, but this trait is independent of photorespiration. The appearance of these abnormal starch granules resembles those that occur in mutant plants defective in the isoamylase-type starch debranching enzymes (ISA), which assemble the starch polymer (Delatte et al., 2006, Wattebled et al., 2005, Zeeman et al., 1998). However, in contrast to the isa mutants that exhibit decreased accumulation of starch and increased accumulation of WSP (Facon et al., 2013, Lin et al., 2013), the mtFAS mutations do not impact starch and WSP accumulation.

The abnormal starch granule trait may be a secondary effect of the deficiency in the photorespiration-independent mtFAS functions, for example the deficiency in lipid A-like molecules, which are enriched within chloroplasts (Li et al., 2011, Armstrong et al., 2006), but are synthesized from mtFAS-derived fatty acids (i.e. 3-hydroxytetradecanoic acid). Considering that alterations in plastid membrane lipid ultrastructure result in abnormal starch granule morphology (Myers et al., 2011), the depletion of lipid A-like molecules in the mtFAS mutants could be causative in the alteration of the change in starch granule morphology.

In summary, this study has further expanded the understanding of the mtFAS system, by biochemically and genetically identifying and characterizing the fourth enzymatic component (i.e. mtHD) of the system. Moreover, by comparing the properties of the mtHD component with the properties of the mtKAS component, we have formulated a more integrated comprehensive understanding of the role of mitochondria in generating acyl chains. These combined genetic and biochemical characterizations of individual components of the Arabidopsis mtFAS system have established that mutations in the mtPPT gene blocks plant development during embryogenesis (Guan et al., 2015).
Mutations in the *mtKAS* gene (Ewald et al., 2007) and *mtMCS* gene (Guan and Nikolau, 2016), and knockdown lines of the *mtPPT* gene (Guan et al., 2015) and *mtHD* gene lead to the diminished lipoylation of the H subunit of GDC (Douce et al., 2001), an enzyme complex essential in photorespiration (Bauwe et al., 2010). The resulting loss of GDC activity manifests a deficiency in photorespiration. Moreover, the characterizations reported herein has expanded the physiological role of mtFAS that in addition to generating the acyl precursor for lipoic acid biosynthesis, mtFAS has a role in determining the complexity of starch granule morphology. However, mechanistically understanding this inter-organelle coordination will require additional molecular, genetic and biochemical studies (Mueller and Reski, 2014).
METHODS

Protein overexpression, substrate syntheses, and in vitro kinetic assays

Genes encoding the Arabidopsis *mtHD*, *mtKAS* and *Streptococcus pneumoniae* ACP synthase (SpACPS) (McAllister et al., 2006) were chemically synthesized by GenScript (Piscataway, NJ) after codon optimization for expression in *E. coli* (See Supplemental Table 1 online for DNA sequences). The *E. coli* *acpP*, *fabD* and *fabG* genes were cloned from wild-type K12 strain (CGSC, Yale University). All six genes were cloned into the pET30b vector (EMD Millipore, Billerica, MA), and the resulting constructs were named *mtHD*-pET (primers H1-H2), *mtKAS*-pET (primers H3-H4), SpACPS-pET (primers H5-H6), *acpP*-pET (primers H7-H8), *fabD*-pET (primers H9-H10), and *fabG*-pET (primers H11-H12) (See Supplemental Table 2 online for primer sequences). These constructs express recombinant proteins with a His-tag located at the C-terminus. Recombinant proteins were expressed in the *E. coli* BL21* strain (Invitrogen, Carlsbad, CA), and purified using Probond Nickel-Chelating Resin (Invitrogen).

For the kinetic assays of the mtHD enzyme, the enoyl-CoA substrates (i.e. trans-Δ^2^-10:1 and trans-Δ^2^-16:1) were synthesized from a chemical reaction between CoA (EMD Millipore, Billerica, MA) and trans-Δ^2^-decenoic acid (TCI Chemicals, Portland, OR) or trans-Δ^2^-dodecenoic acid (Sigma-Aldrich, St. Louis, MO), and purified using a HPLC system (Agilent (Santa Clara, CA) 1200 HPLC system equipped with a Thermo Scientific (Waltham, MA) Hypersil ODS column (250 mm length, 4.0 mm ID, and 5 μm particle size)) as previously describes (Forster-Fromme et al., 2008). Structures of these CoA derivatives were determined using a LC-MS system (Agilent 1100 LC/MSD system) as previously described (Ding et al., 2012). The hydratase activity of mtHD was assayed (for 10 min at 22°C) as previously described (Autio et al., 2008b). Methyl esters that were derived from the reaction products (i.e. 3-hydroxydecanoyl-CoA and 3-hydroxydodecanoyl-CoA) were directly quantified as described previously (Lu et al., 2008).

For the kinetic assays of the mtKAS enzyme, the acyl-ACP substrates were prepared from a SpACPS-catalyzed enzymatic reaction between apo-ACP and acyl-CoAs (Sigma-Aldrich) as described previously (Zhang et al., 2005); the reaction results in the formation of a mixture of acyl- and holo-ACP molecules. The percentage of acyl-ACP in the mixture was determined by MALDI-QTOF analysis. Similarly, holo-ACP was prepared from a reaction of ACP mixture and CoA (EMD Millipore). The kinetic assays for the mtKAS enzyme were performed as previously described (Zhang et al., 2005). Concentrations of three ingredients were optimized (i.e. 50 μM malonyl-CoA, 50 μM holo-ACP, and 100 nM recombinant mtKAS). In addition, 300 μM NADPH and 1 μM recombinant fabG were added in the reaction mix to monitor the reaction (for 5, 10 and 15 min at 22°C) by measuring the consumption of NADPH at 340 nm.

Kinetic values for mtHD and mtKAS are calculated using Prism version 5.0 (GraphPad Software).
**E. coli** strains and lipid A analyses

The Arabidopsis AtLpxD1 (H13-H14) and AtLpxD2 (H15-H16) genes (5' CDS that encodes the putative mitochondrial presequences was removed) were cloned into pBE522 vector (Zhu et al., 2011), resulting in AtlpxD1-pBE and AtlpxD2-pBE. In the presence of AtlpxD1-pBE or AtlpxD2-pBE, the lpxD gene of *E. coli* BL21* strain was replaced by a GenR cassette using a PCR-based method (Datsenko and Wanner, 2000) with primers H17-H18. Hybrid lipid A molecules were extracted from these strains, and analyzed using a LC-MS/MS system (Agilent 1100 LC/MSD system) as described previously (Joo et al., 2012).

Yeast strains and genetic complementation

The yeast *Saccharomyces cerevisiae* strain deficient in the HTD2 gene (YHR067W; BY4741 background; and MATa) was obtained from Thermo Scientific. The yeast HTD2 (Primers H19-H20) and COQ3 mitochondrial presequence (Hsu et al., 1996) (Primers H21-H22) of yeast BY4741 wild-type strain were cloned into YEp351 vector (PGK promoter-driven gene expression) (de Moraes et al., 1995), resulting in HTD2-YEp and YEp351M, respectively. Arabidopsis mtHD (Primers H23-H24) and mtHD_tail (Primers H25-H24) were cloned into YEp351M (5' CDS of 72 bp was replaced by the yeast COQ3 mitochondrial presequence CDS), resulting in mtHD-YEp and mtHD_tail-YEp, respectively. Complementation test was performed as previously described (Autio et al., 2008b).

Plant strains and genetic transformations

The Arabidopsis genetic strains carrying the *mthd-1* (CS856112), *mtkas-2* (SALK_022295), *mtkas-3* (SALK_087186) and *shmt1-2* (SALK_083735) alleles are in Col-0 background, and were obtained from Arabidopsis Biological Resource Center (Columbus, OH; http://abrc.osu.edu). The *atlpxa-1* mutant (WS background) was isolated from the Arabidopsis Functional Genomics Consortium T-DNA mutant population (Krysan et al., 1999). Primers for genotyping are listed as following: H26-H27-H28 for *mtkas-2* and *mtkas-3*; H26-H29-H30 for *shmt1-2*; and H31-H32-H33 for *atlpxa-1*.

For the GFP experiments, the 5' mtHD CDS of 120 bp was cloned into pENTR/D-TOPO vector (Invitrogen; primers H34-H35), and subcloned into pEarleyGate103 (Earley et al., 2006) using Gateway LR Clonase II Enzyme Mix (Invitrogen), resulting in *mtHD*\_120\_pEG. For the RNAi experiment, a mtHD CDS of 209 bp was cloned into pENTR/D-TOPO (primers H36-H37), and subcloned into pB7GIW2G2(II) (Karimi et al., 2002), resulting in *mtHD*-WIWG2. Destination vectors were used to transform Arabidopsis Col-0 wild-type plants (in ambient air) or *mtkas*-2 mutant plants (in the 1% CO₂ atmosphere) as previously described (Clough and Bent, 1998).

Seeds were sterilized and sown on Murashige and Skoog agar medium as previously described (Jin et al., 2012). The CO₂ condition was controlled as ambient level or the 1%
CO₂ level (in a growth chamber). Plants were grown at 22 °C with continuous illumination (photosynthetic photon flux density 100 μmol m⁻² s⁻¹).

**Microscopy**

Confocal microscopy analysis was conducted on the wild-type control plants, the control transgenic plants carrying the p35S::GFP transgene (Guan et al., 2015), and transgenic plants carrying the p35S::HD1-120-GFP transgene. Seedling plants at between 7 to 10 DAI were harvested for analysis as previously described (Guan et al., 2015).

Light microscopy and transmission electron microscopy analyses were performed with plants at 16 DAI using Olympus BX-40 (Olympus America Inc., Center Valley, PA) and JEOL 2100 (Japan Electron Optic Laboratories, Peabody, MA), respectively, as previously mentioned (Myers et al., 2011).

**Western blot**

Total protein was extracted from 200 mg fresh aerial organs of plants at 16 DAI as described previously (Che et al., 2002). Western blot analysis was performed on 50 μg total protein with anti-lipoic acid antibodies (EMD Millipore, Billerica, MA) or with anti-H protein antibodies (a gift from Dr. David Oliver at Iowa State University) as described previously (Ewald et al., 2007, Guan et al., 2015).

**Metabolomic analyses**

Metabolites (3 to 6 replicates) were extracted from 50 mg fresh aerial organs of plants (grown in a completely randomized design) at 16 DAI, and analyzed using multiple analytical platforms: Waters Xevo G2 Q-TOF MS equipped with Waters ACQUITY UPLC system for glycerolipids and chlorophylls (Okazaki et al., 2015); Agilent 1200 HPLC system equipped with a fluorescence detector for amino acids (Guan et al., 2015); the Total Starch Assay Kit (Megazyme, Wicklow, Ireland) for starch and WSP (Smith and Zeeman, 2006); and Agilent 7890 GC-MS system for fatty acids (Lu et al., 2008), surface cuticular lipids (Perera et al., 2010), and other soluble metabolites (Duran et al., 2003). Log₂-ratio and standard error (SE) were calculated as described previously (Quanbeck et al., 2012). Metabolomic data were deposited in the PMR database (Hur et al., 2013) under accession number mtFAS.

**RNA-Seq**

RNA-Seq was performed as previously described (Li et al., 2015). Specifically, total RNA (3 biological replicates) was extracted from 0.5 g fresh aerial organs of plants (grown in a completely randomized design) at 16 DAI using TRIzol (Invitrogen), and was further purified using the RNaseasy Plant Mini Kit (Qiagen, Hilden, Germany) following the DNase I (Invitrogen) treatment to remove any DNA contamination. The 200 bp short-insert library was constructed and sequenced using an Illumina (San Diego, CA) HiSeq 2000 system and V3 Reagent (www.illumina.com). Data files have been deposited in the
NCBI Sequence Read Archive (SRA) under accession numbers SRP052705 and SRP070190.

Raw reads were cleaned by removing adaptors and by filtering reads with unknown nucleotides larger than 5% or with low quality (the percentage of the bases, which have quality smaller than 10, is more than 20% of the read). The cleaned reads were aligned to the reference *Arabidopsis thaliana* genome in Phytozome version 8.0 (www.phytozome.net) using TopHat (Trapnell et al., 2009), and the mapped reads were counted using HTSeq (Anders et al., 2015).

**Statistical analyses**

The count data were analyzed as a completely randomized design with six treatments, one for each combination of genotype and growth condition. Each treatment was replicated three times. Genes with an average of at least one uniquely mapped read across samples and a number of nonzero read counts at least as large as the number of treatments were tested for differential expression among genotypes within each growth condition, differential expression between growth conditions within each genotype, differential expression among genotypes averaged over growth conditions (i.e., genotype main effects), differential expression between growth conditions averaged over genotypes (i.e., growth condition main effects), and interactions between genotypes and growth conditions using the R package QuasiSeq (http://cran.r-project.org/web/packages/QuasiSeq). The negative binomial QLShrink method implemented in the QuasiSeq package as previously described (Lund et al., 2012) was used to compute a *p*-value for each gene and each test described above. The log of each count mean was modeled as the sum of an intercept term, a genotype effect, a growth condition effect, an interaction between genotype and growth condition, and an offset normalization factor, determined for each sample by the log of the TMM normalization factor (Robinson and Oshlack, 2010). Estimates of the fold change, between the *mtkas* mutant and wild-type plants and between ambient air and the 1% CO₂ atmosphere, were computed by evaluating the exponential function at estimates of effect differences. Using the *p*-values for each comparison, an approach previously described (Nettleton et al., 2006) was used to estimate the number of genes with true null hypotheses among all genes tested, and this estimate was used to convert the *p*-values to *q*-values (Storey, 2002). To obtain approximate control of the false discovery rate at 5%, genes with *q*-values no larger than 0.05 were declared to be differentially expressed.

Genes with at least one significant main effect or a significant interaction between genotypes and environmental conditions were divided into seven distinct sets, and a cluster analysis (Fang et al., 2012, Schmollinger et al., 2014, Ramundo et al., 2014) was then performed separately on each set. These sets were defined by each possible combination of the three tests, excluding genes without a significant main effect or interaction effect. For example, one subset is composed of genes with a significant genotype effect, but not a significant environmental condition effect nor a significant
interaction effect. Likewise, another subset consists of genes with a significant environmental condition effect, but not a significant genotype or interaction effect. The remaining five sets similarly each represent a unique combination of genotype, environmental condition, and interaction effects. A model-based clustering algorithm, implemented in the R package MBCluster.Seq (Si et al., 2014), was applied to identify distinct gene expression patterns among the genes in each set. The RNA-seq read counts were assumed to be drawn from a mixture of negative binomial distributions, each of which represents a cluster. After accounting for normalization factors and each gene's overall mean expression level, genes were assigned to clusters based on their expression profiles, or the collection of estimated fold-changes across treatments and genotypes. The number of clusters in each set of genes was chosen using the Akaike information criteria (AIC).

Quantitative RT-PCR

Quantitative RT-PCR was performed using the StepOnePlus Real-Time PCR System and SYBR Select Master Mix (Invitrogen) as previously described (Jin et al., 2012). RNA samples for the expression study were used as previously described (Guan et al., 2015). Gene-specific primers are used as following: H38-H39 for Actin-2 (reference gene; AT3G18780), H40-H41 for mtHD (AT5G60335), H42-H43 for mtKAS (AT2G04540), H44-H45 for RNP1 (AT2G32230), H46-H47 for SQD2 (AT5G01220), H48-H49 for PPH (AT5G13800), H50-H51 for LPAAT (AT4G24160), H52-H53 for DGAT1 (AT2G19450), H54-H55 for CER1 (AT1G02205), H56-H57 for CER2 (AT4G24510), H58-H59 for CER3 (AT5G57800), and H60-H61 for MCCA (AT1G03090).

FIGURE LEGENDS

Figure 1. Comparison of the amino acid sequences of the Arabidopsis mtHD (encoded by AT5G60335) and the human HsHTD2 proteins. Residues shaded in black are identical, those shaded in grey share similarity in the chemistry of the side chains. Alignment was performed using ClustalΩ (AlignX, Vector NTI 10) with a gap opening penalty of 10 and a gap extension penalty of 0.1.

Figure 2. Genetic complementation of the yeast htd2 mutant by the Arabidopsis mtHD gene (AT5G60335). Expression of mtHD was controlled with the phosphoglycerate kinase promoter (pPGK) and terminator (tPGK). Dilution of the inocula for each strain is indicated.

(A) Yeast htd2 mutant strains were grown on glycerol as the sole carbon source. The yeast strain in each row carried the indicated construct: the control empty plasmid (Row 1); construct to overexpress the HTD2 protein (Row 2); construct to overexpress the mitochondrial presequence of the yeast COQ3 protein (MP) (Row 3); and construct to overexpress the mitochondria-targeted mtHD (MP-mtHD fusion protein) (Row 4).
Same strains as in panel A, but they were grown on glucose as the sole carbon source.

Figure 3. Substrate specificity of the recombinant mtHD and mtKAS enzymes.

(A) Substrate concentration dependence of the hydratase reaction catalyzed by mtHD with enoyl-CoA substrates of 10 and 16 carbon atom acyl chain lengths. Michaelis-Menten kinetic parameters tabulated below the graph were calculated from 3 replicates for substrate concentrations of 200 µM, 100 µM and 70 µM; 4 replicates for substrate concentrations of 50 µM and 30 µM; 6 replicates for substrate concentration of 20 µM; and 10 replicates for substrate concentration of 10 µM.

(B) Substrate concentration dependence of the condensation reaction catalyzed by mtKAS with acyl-ACP substrates of between 4 and 16 carbon atom acyl chain lengths. Michaelis-Menten kinetic parameters tabulated below the graph were calculated from 3 replicates for substrate concentrations of 100 µM and 50 µM; 4 replicates for substrate concentrations of 20 µM and 10 µM; and 10 replicates for substrate concentrations of 5 µM and 2 µM.

Figure 4. Subcellular localization of mtHD (encoded by AT5G60335) determined with GFP-tagged transgenes.

(A) Fluorescence micrographs of roots of non-transgenic wild-type control plants (WT); transgenic plants carrying the p35S::mtHD1-120-GFP transgene; and transgenic plants carrying the p35S::GFP control transgene. Confocal fluorescence micrographs imaged the emission of GFP, MitoTracker Orange or the merged images of the GFP and MitoTracker Orange.

(B) Fluorescence micrographs of leaf mesophyll cells of non-transgenic WT; transgenic plants carrying the p35S::mtHD1-120-GFP transgene; and transgenic plants carrying the p35S::GFP control transgene. Confocal fluorescence micrographs imaged the emission of GFP, chlorophyll auto-fluorescence or the merged images of the GFP and chlorophyll auto-fluorescence.

Figure 5. Expression of mtHD (white-filled columns) and mtKAS (grey-filled columns) genes in different organs of Arabidopsis. Quantitative RT-PCR analysis was conducted on RNA templates isolated from different organs of plants at the indicated day after imbibition (DAI). Results are the mean of 3 biological replicates ± standard error, and are presented as normalized values relative to the expression of the Actin-2 gene.

Figure 6. Morphological phenotypes of the mthd-rnai and mtkas mutants.

Single mutants (A) and double mutants (B) were grown in ambient air or in the 1% CO2 atmosphere.

Figure 7. Leaf ultrastructural phenotypes of the mthd-rnai and mtkas mutants.

Light micrographs of leaf cross sections (Column 1), and transmission electron micrographs of chloroplasts (Column 2) and mitochondria (Column 3) of leaf mesophyll
cells of the indicated genotypes, which were grown in ambient air (A) or the 1% CO₂ atmosphere (B).

**Figure 8.** Protein lipoylation status in the aerial organs of the *mthd-rnai* and *mtkas* mutant plants.

(A) Coomassie Brilliant Blue-stained SDS-PAGE analysis of extracts prepared from the indicated genotypes.

(B) Western blot analysis of the H subunit of GDC detected with anti H-protein antibodies, and the lipoylation status of the H-protein and other indicated lipoylated proteins detected with anti-lipoic acid antibodies.

**Figure 9.** Metabolomic alterations in the *mthd-rnai* and *mtkas* mutants.

The *mthd-rnai-1* mutant (A) and *mtkas-2* mutant (B) were grown either in ambient air (black-filled symbols) or in the 1% CO₂ atmosphere (white-filled symbols). The y-axis represents the individual metabolites that were identified. The x-axis plots the log2-transformed, relative ratio of abundance of each metabolite in each mutant sample, normalized to the same metabolite in the wild-type (WT) control sample. Results are presented as mean ± standard error.

**Figure 10.** Effect of the *mthd-rnai* and *mtkas* mutations on the global gene expression profile.

(A) The transcriptomes of the indicated genotypes, grown either in ambient air or in a 1% CO₂ atmosphere were analyzed by RNA-Seq. The 6 comparisons of the transcriptome data of each genotype (arrowed) identified different number of differentially expressed genes (DEGs).

(B) The relative abundances of individual transcripts of DEGs are compared between the mutant and wild-type (WT) plants, grown in either ambient air or in the 1% CO₂ atmosphere. The log2-transformed fold change between the *mthd-rnai-1* mutant and WT plants is plotted against log2-transformed fold change between the *mtkas-2* mutant and WT plants.

**Figure 11.** Clustering and functional annotation analyses of genes that are differentially expressed in response to the two *mtfas* mutations.

(A) Venn diagram representing the classification of differentially expressed genes (DEGs) responding to genotypes (G), environmental conditions (E) and interaction between genotypes and environmental conditions (GxE). Number of DEGs in each set and subset, and the gene expression clusters that belong to each subset (as defined in panel B) are indicated.

(B) Categorization of the expression patterns of 13,514 DEGs among 34 different expression clusters. In each graph the y-axis represents the log2-transformed ratio between the expression value of each condition relative to the mean expression value across all six conditions. Each grey line indicates the expression pattern of an individual
DEG, and the bold black line identifies the average expression pattern of all genes in the
cluster. The x-axis identifies the genotypes of the plants and environmental conditions
under which they were grown. The code for the x-axis ordinances are: WA = wild-type
plants in ambient air; hA = mthd-rnai-1 mutant in ambient air; kA = mtkas-2 mutant in
ambient air; WC = wild-type plants in the 1% CO2 atmosphere; hC = mthd-rnai-1 mutant
in the 1% CO2 atmosphere; and kC = mtkas-2 mutant in the 1% CO2 atmosphere.

(C) Categorization of each gene expression cluster (panel B) is associated with one of
five biological functions. These functions were heuristically determined from the average
differential expression pattern defined by each expression cluster.

SUPPLEMENTAL DATA

Supplemental Figure 1. Mass spectrometric characterization of the acyl-CoA and acyl-
ACP substrates synthesized for the in vitro enzymological assays of mtHD- and mtKAS-
catalyzed reactions.

Supplemental Figure 2. Characterizations of the mthd-1 mutant allele (CS856112).

Supplemental Figure 3. Metabolomic alterations in the mthd-rnai-2 and mtkas-3
mutants.

Supplemental Figure 4. Metabolic alterations in the mtkas-2-mthd-rnai double mutants.

Supplemental Figure 5. Morphological (A), ultrastructural (B) and metabolomic (C)
alterations in response to the feeding of 3% sucrose or 10 mM glycolate.

Supplemental Figure 6. Mass spectrometric characterization and identification of 3-
hydroxytetradecanoic acid and lipid A.

Supplemental Figure 7. Validation of RNA-Seq gene expression data by quantitative
RT-PCR analysis.

Supplemental Figure 8. GO enrichment analysis of differentially expressed genes
associated with the mtFAS biological function.

Supplemental Table 1. Codon-optimized synthetic gene sequences.

Supplemental Table 2. Nucleic acid sequences of DNA primers used in this study.
Restriction enzyme cleavage sites are indicated in the yellow background.

Supplemental Data 1. Statistical test for DEGs among treatment groups. The q-value
and fold change (on a logarithmic scale; base 2) are indicated.

Supplemental Data 2. Gene counts associated with each GO functional category (row)
in each comparison group (column).

Supplemental Data 3. Statistical test for genes whose expressions are associated with
main effects (i.e. G, E and GxE). Seven subsets are shown in column B.
Supplemental Data 4. GO terms enriched in each gene cluster relative to biological processes, subcellular components and molecular functions.

ACKNOWLEDGEMENTS

The authors acknowledge Tracey Stewart and Randall Den Adel of the Microscopy and NanolImaging Facility (Iowa State University, Ames, IA) for the light microscopy and transmission electron microscopy analyses; Dr. Jiqing Peng of the Protein Facility (Iowa State University) for characterization of ACP derivatives; BGI Americas (Cambridge, MA) for library construction and RNA-Seq; Kouji Takano (RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa, Japan) for technical assistance in obtaining the lipidome data; Drs. Manhoi Hur and Eve Wurtele (Iowa State University) for depositing metabolomics data into the PMR database; Drs. Wei Zhang, Peng Liu and Wei Fang (Iowa State University) for help in the RNA-Seq data analysis; Drs. Alan Myers, Thomas Bobik and Young-Jin Lee (Iowa State University) for technical support; Drs. Lloyd Sumner (University of Missouri, Columbia, MO) and Richard Dixon (University of North Texas, Denton, TX) for helpful discussions; and the WM Keck Metabolomics Research Laboratory and the Confocal Microscopy Facility (Iowa State University) for providing access to instrumentation in the metabolomic analyses and subcellular localization studies, respectively. This work was partially supported by the National Science Foundation (Awards IOS1139489, EEC0813570 and MCB0820823 to B.J.N.), the State of Iowa, the Japan Science and Technology Agency Strategic International Collaboration Research Program (SICORP), and RIKEN Pioneering Project Integrated Lipidology.


FORSTER-FROMME, K., CHATTOPADHYAY, A. & JENDROSSEK, D. 2008. Biochemical characterization of AtuD from Pseudomonas aeruginosa, the first member of a new subgroup of acyl-CoA dehydrogenases with specificity for citronellyl-CoA. Microbiology, 154, 789-96.


GUAN, X. & NIKOLA, B. J. 2016. AAE13 encodes a dual-localized malonyl-CoA synthetase that is crucial for mitochondrial fatty acid biosynthesis. Plant J, 85, 581-93.


MEYER, E. H., HEAZLEWOOD, J. L. & MILLAR, A. H. 2007. Mitochondrial acyl carrier proteins in Arabidopsis thaliana are predominantly soluble matrix proteins and none can be confirmed as subunits of respiratory Complex I. Plant Mol Biol, 64, 319-27.


ZHANG, M., FAN, J., TAYLOR, D. C. & OHLROGGE, J. B. 2009. DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. Plant Cell, 21, 3885-901.