Running Title: Systematic phenotypic screen of peroxisomal mutants

To whom correspondence should be addressed: Jianping Hu, MSU-DOE Plant Research Laboratory, 612 Wilson Road, Michigan State University, East Lansing, MI 48824. Email: huji@msu.edu. Phone: 517-432-4620.

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Systematic phenotypic screen of *Arabidopsis* peroxisomal mutants identifies proteins involved in β-oxidation

Gaëlle Cassin-Ross¹ and Jianping Hu¹,²

¹ Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.
² Plant Biology Department, Michigan State University, East Lansing, MI 48824.

**One-sentence Summary:** A systematic phenotypic analysis of mutants of recently discovered *Arabidopsis* peroxisomal proteins identified proteins involved in β–oxidation.

**Keywords:** peroxisomes, reverse genetics, β-oxidation, sugar dependence, 2,4-DB, OPDA, seed germination, fatty acids
Footnotes:

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Corresponding author: Jianping Hu, email: huji@msu.edu.
ABSTRACT
Peroxisomes are highly dynamic and multi-functional organelles essential to development. Plant peroxisomes accommodate a multitude of metabolic reactions, many of which are related to the β-oxidation of fatty acids or fatty acid-related metabolites. Recently, several dozens of novel peroxisomal proteins have been identified from Arabidopsis through in silico and experimental proteomic analyses followed by in vivo protein targeting validations. To determine the functions of these proteins, we interrogated their T-DNA insertion mutants with a series of physiological, cytological and biochemical assays to reveal peroxisomal deficiencies. Sugar-dependence and 2,4-dichlorophenoxybutyric acid (2,4-DB) and 12-oxo-phytodienoic acid (OPDA) response assays uncovered statistically significant phenotypes in β-oxidation-related processes in mutants for 20 out of the 27 genes tested. Further investigations uncovered a subset of these mutants with abnormal seed germination, accumulation of oil bodies, and delayed degradation of long-chain fatty acids during early seedling development. Mutants for seven genes exhibited deficiencies in multiple assays, strongly suggesting the involvement of their gene products in peroxisomal β-oxidation and initial seedling growth. Proteins identified included isoforms of enzymes related to β-oxidation, such as Acyl-CoA thioesterase 2 and Acyl-activating enzyme isoform 1 and 5, and proteins with functions previously unknown to be associated with β-oxidation, such as Indigoidine synthase A, Senescence-associated protein/B12D-related protein 1, Betaine aldehyde dehydrogenase, and Unknown protein 5. This multi-pronged phenotypic screen allowed us to reveal β-oxidation proteins that have not been discovered by single-assay-based mutant screens, and enabled the functional dissection of different isoforms of multigene families involved in β-oxidation.
INTRODUCTION

Peroxisomes are small (~0.1–1.0 µm) and single-membrane eukaryotic organelles that are essential for the development of animals and plants by mediating a multitude of conserved and lineage-specific metabolic functions (Beevers, 1979; Van den Bosch et al., 1992; Kaur et al., 2009; Hu et al., 2012; Schrader et al., 2012). Plant peroxisomes house metabolic processes including \(\beta\)-oxidation of fatty acids, hormone biosynthesis, photorespiration, the glyoxylate cycle, detoxification of reactive oxygen, nitrogen and sulfur species (ROS, RNS, and RSS), and metabolism of branched amino acids, urate and polyamines (Beevers, 1979; Kaur et al., 2009; Hu et al., 2012). Peroxisomes also generate signaling molecules with regulatory roles in plant development (Weber, 2002; Corpas et al., 2013; Sandalio et al., 2013).

\(\beta\)-oxidation of fatty acids and related metabolites is a major function of peroxisomes throughout the life cycle of a plant, from seed germination to senescence. Mobilization of seed oil reserves during seed germination and post-germinative growth requires peroxisomal \(\beta\)-oxidation and the glyoxylate cycle. In this process, fatty acids are transported into the peroxisome, where they are activated into fatty acyl-CoAs and later shortened by two carbons in each cycle of \(\beta\)-oxidation. The product, acetyl-CoA, is converted to 4-carbon molecules by the glyoxylate cycle, whose products further undergo gluconeogenesis to provide energy for post-germinative development (Theodoulou and Eastmond, 2012). Using core \(\beta\)-oxidation enzymes as well as pathway-specific enzymes, 12-oxo-phytodienoic Acid (OPDA), the jasmonic acid (JA) precursor that enters the peroxisome after being synthesized in the chloroplast, is converted to JA (Acosta and Farmer, 2010), and indole 3-butyric acid (IBA) is converted to the principal form of auxin, indole 3-acetic acid (IAA) (Strader and Bartel, 2011). Besides the core \(\beta\)-oxidation pathway, which metabolizes straight-chain saturated fatty acids, auxiliary \(\beta\)-oxidation pathways also occur in the peroxisome to metabolize unsaturated fatty acids, in which case accessory enzymes are required (Goepfert and Poirier, 2007; Graham, 2008).

To assess the full composition of this versatile organelle in plants, both \textit{in silico} analysis and experimental proteomics have been employed to identify novel peroxisomal proteins. Bioinformatic analysis of the \textit{Arabidopsis} genome using Peroxisomal Target Signal type 1 (PTS1) and PTS2 sequences predicted a total of over 400 proteins to be potentially peroxisomal
(Lingner et al., 2011). Experimental proteomics of Arabidopsis, spinach and soybean peroxisomes using different tissue/cell types and development stages together identified several dozens of novel peroxisomal proteins after in vivo targeting verification (Fukao et al., 2002; Fukao et al., 2003; Arai et al., 2008, 2008; Eubel et al., 2008; Reumann et al., 2009; Babujee et al., 2010; Quan et al., 2013). Following the identification of peroxisomal proteins from etiolated Arabidopsis seedlings through proteomics and in vivo protein targeting analysis, we used reverse genetics to analyze the mutants of five newly identified proteins and revealed the role of a cysteine protease, RDL1 (RESPONSE TO DROUGHT21A-LIKE1), in seed germination, β-oxidation, and stress response (Quan et al., 2013; Cassin-Ross and Hu, 2014). However, many other recently identified peroxisomal proteins have not been characterized with respect to their functions in peroxisomal physiology and plant development.

In this study, we interrogated the mutants of 27 recently identified peroxisomal genes with systematic phenotypic assays to analyze the function of the proteins in peroxisomal metabolism. Mutants for 20 of the tested genes showed statistically significant phenotypes in at least one of the assays. Further analysis revealed that mutants for seven of the 20 genes displayed deficiencies in multiple assays, suggesting strongly that these seven proteins are involved in β-oxidation-related processes. This multi-faceted screen enabled us to identify β-oxidation proteins that may not have been discovered otherwise by genetic screens based on single assays.

RESULTS

Identifying mutants of recently discovered Arabidopsis peroxisomal genes
To investigate the role of the recently identified and uncharacterized peroxisomal proteins in peroxisomal physiology, we took a reverse genetic approach by searching the TAIR database (http://www.Arabidopsis.org) for T-DNA insertion lines. Most of these proteins were identified from our own proteomic analyses of Arabidopsis peroxisomes from green leaves and etiolated seedlings (Reumann et al., 2009; Quan et al., 2013), and some were identified by other research groups. A few of the genes had been previously investigated in processes not directly related to β-oxidation, and a few others had been studied biochemically without mutant characterization. These included Calcium-dependent protein kinase 1 (CPK1) and Hydroxy-acid oxidase isoform
2 (HAOX2) that function in innate immunity (Coca and San Segundo, 2010; Rojas et al., 2012), 1,4-dihydroxy-2-naphthoyl thioesterase 1 (DHNAT1) that is involved in DHNA-CoA hydrolysis in phylloquinone biosynthesis (Widhalm et al., 2012), Acetoacetyl CoA thiolase 1 (AACT1) responsible for the condensation of two acetyl-CoA molecules to form acetoacetyl CoA (Jin et al., 2012), and Copper amine oxidase 3 (CuAO3) in polyamine catabolism (Planas-Portell et al., 2013).

We obtained seed stocks of 53 putative mutant lines for 30 peroxisomal genes, and genotyped these lines by PCR analysis of genomic DNA followed by sequencing of the PCR products. Despite numerous efforts, we were unable to confirm T-DNA insertion in six lines for three genes (Supplemental Table S1). In addition, homozygous plants could not be identified from seven lines (all in Col-0 background) for five peroxisomal proteins, i.e. NADP-dependent isocitrate dehydrogenase (ICDH), Uricase (URI), Phosphogluconate dehydrogenase (6PGDH), Senescence-associated protein (B12D1), and Alanine:glyoxylate aminotransferase 2 (AGT2) (Supplemental Table S1). We predicted that homozygous mutants for these five genes may be embryonic or seedling lethal in Col-0 background. Consistent with the notion that the null mutant of ICDH, an enzyme that regenerates NADPH, may be lethal, homozygous null mutants for two genes in the plastid-peroxisome dual-localized oxidative pentose phosphate pathway, which also generates NADPH, were found to be non-viable as well (Xiong et al., 2009; Bussell et al., 2013). These data support the idea that NADPH regeneration is essential for plant survival. Finally, the lethal phenotype seems to be ecotype-specific for B12D1, as its null mutant was viable in Ws-4 background.

A total of 40 homozygous T-DNA insertion mutants for 27 genes were obtained; at least two mutant alleles were available for 12 genes (Table 1; Supplemental Figure S1). We performed RT-PCR analysis to detect the level of full-length transcripts of the target genes, and discovered that 30 mutants for 23 peroxisomal genes were possible null alleles (Supplemental Figure S2). In addition, the expression level of acyl-CoA thioesterase 2 (ACH2), 2,4-dienoyl-CoA reductase (DECR), Epoxide hydrolase isoform 3 (EH3), and two genes encoding unknown proteins (UP5 and UP7) was decreased in ach2-3, decr-3, eh3-1, up5-1 and up7-2 respectively, suggesting that these mutants were knock-down alleles. On the other hand, the expression level of ICDH,
calcium binding EF-hand (CEF), acyl transferase 2 (ATF2), DHNA-CoA thioesterase 1 (DHNAT1) and small thioesterase 5 (sT5) was increased in icdh-1, cef-1, atf2-1, dhnat1-2 and st5-2 respectively, indicating that the target genes were overexpressed in these lines (Supplemental Figure S2). All these viable homozygous lines, including both loss-of-function mutants and overexpression lines, were used in subsequent analyses.

Except for CPK1 and AACT1, 25 of the peroxisomal proteins analyzed in this study were initially identified from the leaf peroxisome proteome (Supplemental Table S2), indicating that they may be involved in photorespiration or general plant growth. Photorespiration is a major peroxisomal function in green tissue, where peroxisomes together with chloroplasts and mitochondria convert phosphoglycolate produced by the oxygenase activity of Ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO) into glycerate, a molecule that is re-used in the chloroplastic Calvin-Benson cycle (Foyer et al., 2009; Peterhansel et al., 2010). Apart from the control mutant pex14, which is defective in peroxisomal protein import, none of the mutants showed obvious difference in appearance from the wild type while growing under ambient air (CO₂ 400 µL L⁻¹) or decreased levels of CO₂ (80 µL L⁻¹), the latter of which was expected to enhance the growth defect of photorespiratory mutants because of the higher demand for photorespiration under low CO₂ (Supplemental Figure S3). We concluded that these proteins are not essential for plant growth or photorespiration, or they play redundant roles with other proteins in these processes under our laboratory conditions. Since the majority of the peroxisomal proteome is dedicated to β-oxidation-related processes (Hu et al., 2012), we next employed parallel physiological assays to assess the efficiency of peroxisomal β-oxidation in these mutants.

**Mutants for four genes were sugar-dependent in post-germinative growth**

Prior to the establishment of photosynthesis, peroxisomal fatty acid β-oxidation is required to fuel early seedling growth. As a result, many β-oxidation mutants arrest or develop slowly after germination, a phenotype that can be rescued by adding to the growth medium a carbon source, such as sucrose (Suc) (Hu et al., 2012). To check for Suc dependence, we grew mutants for seven days on half-strength Linsmaier and Skoog (1/2 LS) medium in dark or light conditions with or without Suc, and quantified hypocotyl or root lengths. Only differences between mutants
and wild-type plants with a p value <0.01 were considered statistically significant after an unpaired Student’s t test.

As expected, pex14 displayed sugar-dependent growth in both dark and light conditions (Figure 1). Although not showing sugar dependence, dhar1, icdh, atf2, b12d1 and cpk1 had statistically shorter or longer hypocotyls and/or roots than the wild-type plants regardless of the presence of Suc in dark or light conditions (Figure 1). Since the size of these mutants was indistinguishable from that of the wild type as adults (Supplemental Figure S3), we reasoned that the developmental differences observed at the seedling stage were possibly overcome later.

In the dark, the absence of Suc resulted in a ~15% decrease in the hypocotyl length of wild-type Col-0 and most mutant seedlings, whereas the decrease was ~26% for icdh-1 and 21% for up5-1 (Figure 1A, Supplemental Table S3). In light-grown seedlings, the absence of Suc caused a ~16% reduction of the primary root length in Col-0 and most mutants, whereas this reduction was ~24% for both inda-1 and up7-1 (Figure 1B, Supplemental Table S3). These results indicated possible roles of ICDH and UP5, and INDA and UP7, in lipid mobilization during post-germinative growth in dark and light-grown conditions respectively.

Mutants for 14 genes showed abnormal 2,4-DB response

IBA is converted to IAA through peroxisomal β-oxidation (Strader and Bartel, 2011). To check whether any mutants showed reduced response to the inhibitory effect of IBA on primary root elongation, root lengths of 7d seedlings grown on 1/2 LS medium supplemented with IBA or IAA were measured. For wild-type and mutants, control treatment with 100 nM IAA resulted in similar degrees of decreases in the primary root length coupled with excessive root hair growth (Supplemental Figure S4). The pex14 mutant seedlings exhibited strong IBA resistance, yet all the other mutants showed IBA response similar to that of the wild type (Supplemental Figure S4 and S5) with no statistically significant differences observed at tested IBA concentrations (Student’s t test, p<0.01).

Given that 2,4-dichlorophenoxybutyric acid (2,4-DB) is converted to the synthetic auxin analogue 2,4-dichlorophenoxy acetic acid (2,4-D) via β-oxidation (Hayashi et al., 2000), we
studied the response of the mutants to 2,4-DB and 2,4-D. Wild-type plants and the tested mutants showed similar levels of response to 100 nM 2,4-D, *i.e.* shortened primary root length and excessive root hairs (Supplemental Figure S4). While growing on 2,4-DB, wild-type seedlings showed a heterogeneous response (Supplemental Figure S6). Therefore, we instead scored the percentage of 2,4-DB resistant seedlings, which were defined as having root length statistically the same as that of untreated seedlings. Treatment with 0.4, 0.8 and 1 μM of 2,4-DB resulted in a decrease of the primary root length in ~30%, 65% and 67% of the wild-type Col-0 seedlings, but only in ~8%, 24%, and 26% of wild-type Ws-4 seedlings (Figure 2). Because wild-type Ws-4 and Col-0 plants showed similar sensitivity to 2,4-D (Supplemental Figure S4), we speculated that Ws-4 plants were less sensitive to 2,4-DB and therefore assessed the response of mutants in Ws-4 background with higher concentrations of 2,4-DB. Sixty-six percent and 95% of wild-type Ws-4 seedlings were sensitive to 1.5 and 2 μM 2,4-DB respectively (Supplemental Figure S7).

As shown in Figure 2 and Supplemental Figure S7, *dhnat1-1* and *elt1-1* had statistically lower percentage of resistant seedlings in comparison to wild-type plants, and were therefore considered hypersensitive to 2,4-DB. In contrast, *ach2, up6-1, dhar1, icdh-1, b12d1, badh-1, cadc, eh3-2, cpk1, aae5-1, up5-1* and *aact1-1* showed statistically higher percentage of resistant seedlings than wild type under at least two concentrations of 2,4-DB (Student’s *t* test, *p*<0.01), and were considered having reduced 2,4-DB response. Taken together, these results suggested that DHNAT1, ELT1, ACH2, UP6, DHAR1, ICDH, B12D1, BADH, CADC, EH3, CPK1, AAE5, UP5 and AACT1 may be involved in 2,4-DB metabolism. The fact that *ICDH* was overexpressed in *icdh-1* implied that ICDH may play a negative role in 2,4-DB catabolism.

**Mutants of nine genes showed deficiency in OPDA metabolism**

Methyl-JA (MeJA) inhibits primary root elongation (Staswick et al., 1992). We reasoned that the JA precursor 12-oxo-phytodienoic acid (OPDA), which is converted to JA through peroxisomal β-oxidation, would similarly inhibit primary root elongation. Based on this assumption, we previously developed a simple assay to identify new peroxisomal proteins involved in OPDA metabolism (Cassin-Ross and Hu, 2014). The same assay was applied in this study to assess the function of the tested proteins in OPDA metabolism.
Compared with the wild type, the control mutant \textit{opcl1}, which is defective in OPC-8:0 CoA Ligase1 in JA biosynthesis (Koo et al., 2006), showed resistance to OPDA’s inhibition on primary root elongation, whereas MeJA exerted similar effect on \textit{opcl1} and wild-type plants, suggesting that \textit{opcl1} was specifically resistant to OPDA (Figure 3). Except for \textit{aae1-I}, all other mutants responded to MeJA similarly to the wild type \textit{i.e.} ~60\% inhibition of root elongation (Figure 3). The primary root of \textit{ach2}, \textit{cuao3-I}, \textit{decr-2} and \textit{cpk1-I} showed reduced OPDA response similar to \textit{opcl1}, whereas \textit{up6-I}, \textit{aae1-I}, \textit{dhnat1-I}, \textit{st5} and \textit{eh3-I} appeared to show enhanced response to OPDA (Student’s \textit{t} test, \textit{p}<0.01, Figure 3). These results suggested that \textit{ACH2}, \textit{CUAO3}, \textit{DECR}, \textit{UP6}, \textit{AAE1}, \textit{DHNAT1}, \textit{ST5}, \textit{EH3}, and \textit{CPK1} may affect the catabolism of OPDA.

\textbf{Most proteins with \(\beta\)-oxidation-related phenotypes are involved in seed germination}

Our sucrose dependence and 2,4-DB/OPDA response assays together identified 31 mutants for 20 peroxisomal genes that showed a phenotype in at least one of the assays; 10 of the genes had at least two alleles with similar phenotypes. These data suggested the potential roles of \textit{AACT1}, \textit{AAE1}, \textit{AAE5}, \textit{ACH2}, \textit{B12D1}, \textit{BADH}, \textit{CADC}, \textit{CPK1}, \textit{CUAO3}, \textit{DECR}, \textit{DHAR1}, \textit{DHNAT1}, \textit{EH3}, \textit{ELT1}, \textit{INDA}, \textit{ICDH}, \textit{ST5}, \textit{UP5}, \textit{UP6}, and \textit{UP7}, in \(\beta\)-oxidation. Since fatty acid \(\beta\)-oxidation plays a key role in seed germination and early post-germinative growth (Theodoulou and Eastmond, 2012), we first determined whether these 20 proteins are involved in seed germination. Positive controls used in this experiment and subsequent assays were previously characterized null mutants \textit{acx4-I} and \textit{kat2-3}, which are impaired in genes encoding the core \(\beta\)–oxidation enzymes acyl-CoA oxidase 4 (ACX4) and 3-ketoacyl CoA thiolase 2 (KAT2) respectively (Germain et al., 2001; Rylott et al., 2003).

Fresh seeds from all 30 mutants germinated normally, as quantified by radicle emergence from seeds grown on plain agar (0.8\% agar) or 1/2 LS medium (Supplemental Figure S8). We then tested seed germination in response to factors known to influence this process. Light is a positive regulator of seed germination (Lau and Deng, 2010), so we compared the germination rate of mutant seeds that were placed in total darkness with those that were subjected to 1h light treatment before germination. Quantification of radicle emergence from 5d seedlings revealed that, while wild-type Col-0, Col-3 and the positive control \textit{kat2-3} showed no light dependence,
wild-type Ws-4 appeared to depend more on the light treatment for germination (Figure 4). Together with acx4-1, mutants for 19 of the 20 genes tested showed statistically lower germination rate in comparison with their respective wild-type controls when germinated in total darkness (Student’s t test, p<0.01), and the 1h light pre-treatment could rescue the germination potential for all these mutants except for inda-1, b12d1-1, and elt1-1 (Figure 4).

We also quantified seed germination rate on medium supplemented with the phytohormone abscisic acid (ABA), which acts synergistically with OPDA to inhibit germination (Vanstraelen and Benkova, 2012). Radicle emergence was quantified on seeds sown on plain agar supplemented with 2 or 5 µM ABA (Figure 5). As previously reported, kat2-3 exhibited insensitivity to ABA regardless the concentration (Jiang et al., 2011). However, germination rate of acx4-1 was statistically lower than the wild type, and similarly, most of the 5d or 10d mutant seedlings showed statistically significant hypersensitivity to 2 µM ABA in comparison with the wild type (Student’s t test, p<0.001). At 5 µM ABA, all the 5d seeds except b12d1-1 showed hypersensitive response, whereas at 10d the germination rate for ach2, up6, dhar1, cuao3, cpk1 and aae5 were comparable to the wild type (Figure 5).

Based on results from these two germination assays, we concluded that most of these 20 proteins have potential positive roles in initial seed germination and the role for INDA and B12D1 seemed more prominent given the stronger phenotypes of their mutants. To determine whether the seed germination phenotypes correlated with gene expression pattern, we analyzed the expression of the 20 genes during seed maturation and germination. Using data from the publicly available Botany Array Resource database (Toufighi et al., 2005), we generated a heatmap using log2-normalized data of the relative expression for 18 of these genes (data for INDA and UP7 were unavailable). During some stages of seed maturation and germination, UP6, DECR, EH3, AACT1, and AAE5 were up-regulated whereas DHAR1, ACH2, sT5, DHNAT1, AAE1, CuAO3, ICDH, BADH, ELT1 and B12D1 were repressed to various levels. Consistent with the relatively strong germination phenotype of the b12d1 mutant, the expression of B12D1 is markedly increased during the seed germination process (Supplemental Figure S9, Supplemental Table S4).
Mutants for seven peroxisomal proteins retain oil bodies and accumulate fatty acids in early seedling development

Previous studies have shown that the inability to break down triacylglycerol (TAG) leads to prolonged presence of oil bodies in β-oxidation mutants (Graham, 2008). To assess lipid mobilization defects in the peroxisomal mutants, we used the lipophilic stain nile red (Greenspan et al., 1985) to detect the presence of lipid bodies in hypocotyls of 5d and 7d etiolated seedlings. Fluorescence microscopy revealed that 5d after germination, both wild-type and mutant hypocotyls contained abundant oil bodies (Supplemental Figure S10), which were markedly diminished at 7d in the wild type and most mutants (Supplemental Figure S11). In contrast, the two positive controls acx4-1 and kat2-3 and seven mutants, i.e. ach2-3, aae1-1, inda-1, b12d1-1, badh-1, aae5-1 and up5-1 still retained a significant number of oil bodies 7d after germination (Figure 6).

To confirm the oil body accumulation phenotype in these seven mutants, we quantified long-chain fatty acid species and compared their levels in 3d, 5d, and 7d etiolated seedlings against seeds. At 3d and 5d after germination, the amount of C20:1, which is a marker for TAG (Lemieux et al., 1990), remained at a statistically higher level in virtually all the mutants than in wild-type seedlings (Figure 7A and Figure 7B). Similarly, higher accumulation of other long-chain fatty acids, i.e. C16:0, C18:0, C18:1, C18:2, C18:3, C20:2, and C20:3, was observed in nearly all the mutants at 3d and in all the mutants at 5d (Figure 7A and Figure 7B; Student’s t test, p<0.01). Consistent with the strongest retention of oil bodies found in this study (Figure 6), kat2-3 showed the highest accumulation of all long-chain fatty acids analyzed (Figure 7). At 7d after germination, the level of long-chain fatty acids was still higher in kat2-3 and inda-1 but comparable or even lower in other mutants in comparison with the wild type (Figure 7C). These data support the notion that ach2-3, aae1-1, inda-1, b12d1-1, badh-1, aae5-1 and up5-1 had a reduced rate of β-oxidation, which caused delayed degradation of the fatty acid substrates.

DISCUSSION

Multipronged phenotypic screen allows the identification of additional peroxisomal proteins potentially involved in β-oxidation
Forward genetic screens for mutants with sugar dependence or resistance to 2,4-DB/IBA identified key enzymes in β-oxidation and proteins involved in peroxisome biogenesis (Hu et al., 2012). However, these screens rely on single assays and tend to isolate mutants with strong visual phenotypes. In this study, we used multiple assays to simultaneously screen mutants of 27 uncharacterized peroxisomal genes in a quantitative manner. Mutants of 20 genes showed statistically significant phenotypes in β-oxidation-related processes, and further investigations discovered a subset of them with abnormal seed germination, accumulation of oil bodies, and delayed degradation of long-chain fatty acids during early seedling development. Mutants for seven genes exhibited deficiencies in multiple β-oxidation-based assays, strongly suggesting the involvement of these proteins in peroxisomal β-oxidation. ACH2, AAE1 and AAE5 are isoforms of enzymes known to be related to β-oxidation, whereas INDA, B12D1, BADH, and UP5 belong to functional categories previously unknown to be associated with β-oxidation, and their roles in this pathway could be direct or indirect. This screen provided a complementary approach to previous genetic screens in the identification of β-oxidation proteins that may have not been uncovered by single-assay-based screens. In addition, although β-oxidation occurs throughout the life cycle of a plant, many mutants in this pathway do not show obvious phenotypes at mature stages (Hu et al., 2012). Therefore, using assays aimed at dissecting β-oxidation in initial plant development, this study was able to unmask phenotypes for several mutants and will help us understand the β-oxidation network in more depth.

**ACH2, an acyl-CoA thioesterase involved in the hydrolysis of long-chain fatty acyl-CoAs**

Acyl-CoA thioesterases (ACOTs) hydrolyse fatty acyl-CoAs, yielding free fatty acids and coenzyme A (CoASH). The complex role of these enzymes in lipid metabolism has been previously documented, in particular in animals (Hunt et al., 2012). Mammalian ACOT8, the closest homologue to the two *Arabidopsis* peroxisomal ACOTs (ACH1 and ACH2), showed high activities toward a broad range of acyl-CoA substrates and was strongly inhibited by CoASH, suggesting that ACOT8 is involved in the regulation of the intracellular levels of acyl-CoAs, free fatty acids and CoASH (Hunt et al., 2002; Ofman et al., 2002).

ACH2 was the first acyl-CoA thioesterase to be cloned from plants, and its recombinant protein showed high levels of acyl-CoA thioesterase activity against both medium and long-chain fatty acids.
acyl-CoAs, with the highest activity toward long-chain unsaturated fatty acyl-CoAs (Tilton et al., 2000; Tilton et al., 2004). We have shown in this study that loss-of-function ach2 mutants have higher accumulation of long-chain fatty acids in germinated seedlings and partial resistance to 2,4-DB and OPDA, supporting ACH2’s positive role in β-oxidation. Interestingly, the ACH2 knock-down allele ach2-3 displayed a stronger seed germination phenotype than the null alleles, possibly due to functional compensation among multigene family members in the null alleles. Our study has expanded the list of potential substrates for ACH2, suggesting that it may be involved in the regulation of the intracellular level of acyl-CoAs, acid-CoAs (OPDA and 2,4-DB), free fatty acids, acids and CoASH. However, ACH2’s high expression in mature tissues such as young leaves and flowers and low expression in germinating seedlings (Tilton et al., 2004), the relatively weak phenotypes observed in ach2 mutants in seed germination, and the fact that ach2 mutant was never isolated from previous forward genetic screens, together suggest that ACH2 may not play a major role during early seedling development.

Two additional isoforms of acyl-activating enzymes involved in early seedling development

Arabidopsis AAE1 and AAE5 belong to a 14-member plant specific clade in the acyl-activating enzymes superfamily (Shockey et al., 2003). Some proteins from this clade have been reported to contain acid:CoA ligase activities. Benzoyloxyglucosinolate 1 (BZO1) possesses benzoyl-CoA ligase activity (Kliebenstein et al., 2007), and AAE7 and AAE11 have acyl-CoA synthetase activity towards short- and medium-chains respectively (Shockey et al., 2003). However, the function and/or substrate specificity of AAE1 and AAE5 and that of many other AAEs, are still unknown.

It was reported that AAE1 is not involved in the activation of short to medium-chain acid substrates ranging in length from C2 (acetate) to C14 (myristate) (Shockey et al., 2003). We have shown in this study the accumulation of oil bodies and delayed degradation of long-chain fatty acids in aae1-1 etiolated seedlings. In addition, aae1-1 is hypersensitive to ABA and OPDA, two hormones required for dormancy. These results suggest that during seed germination and early seedling development, AAE1 is involved in the peroxisomal activation of long-chain fatty acids before these substrates enter the β–oxidation cycle.
In our study, loss-of-function mutant of AAE5 showed a weaker phenotype than that of AAE1, where aae5-1 exhibited partial resistance to 2,4-DB, and weak accumulation of oil bodies and delayed degradation of long-chain fatty acids in etiolated seedlings. These results suggest that AAE5 may be involved in the activation of long-chain fatty acids in Arabidopsis but its role is not as prominent as AAE1 in germination and early seedling development. AAE1 is ubiquitously expressed throughout the plant whereas the expression of AAE5 is limited to developing seeds and roots (Shockey et al., 2003). In light of its high expression in developing seeds, a tissue in which the flow of fatty acids can be adjusted via β-oxidation (Poirier et al., 1999), we speculate that AAE5 may play a stronger role in activating long-chain fatty acids in developing seeds.

Taken together, our assays enabled us to distinguish the function of AAE1 and AAE5, two proteins that encode enzymes with the same biochemical activity and had been considered functionally redundant.

A betaine aldehyde dehydrogenase involved in β-oxidation

BADH belongs to subfamily 10 of the aldehyde dehydrogenase (ALDH) family, which is composed of amino-aldehyde dehydrogenases (AMADHs; EC 1.2.1.19), 4-aminobutyraldehyde dehydrogenases, 4-guanidinobutyryl-aldehyde dehydrogenases (EC 1.2.1.54), and betaine aldehyde dehydrogenases (BADHs; EC 1.2.1.8) (Brocker et al., 2013). Based on findings that BADH is induced by ABA, dehydration, salt, chilling and oxidative stresses and that BADH oxidizes betaine aldehyde, 4-aminobutyraldehyde and 3-aminopropionaldehyde to their corresponding carboxylic acids, it has been suggested that BADH may serve as a detoxification enzyme essential for the control of the level of aminoaldehydes that are produced in response to abiotic stresses (Missihoun et al., 2011). A recent study suggested that BADH, in conjunction with CuAO2 and CuAO3, functions in polyamine (PA) homeostasis (Planas-Portell et al., 2013). Similarly to BADH, the expression CuAO3 is also increased in response to ABA, MeJA, SA and flagellin, suggesting that PA catabolism may be involved in plant stress response (Planas-Portell et al., 2013). In addition, PAs are essential for a wide range of physiological processes, including seed germination (Pieruzzi et al., 2011).
We have demonstrated that plants lacking BADH are hypersensitive to ABA’s inhibitory effect on germination, accumulate higher amounts of long-chain fatty acids and oil bodies days after germination, and exhibit 2,4-DB resistance. These data point to a positive role for BADH in β-oxidation and seed germination. BADH’s role in β-oxidation remains to be determined.

An indigoidine synthase in seed germination
INDA is involved in the biosynthesis of indigoidine, a blue pigment first described in the bacterium *Erwinia chrysanthemi* and is implicated in protecting bacteria against oxidative stresses generated by reactive oxygen species produced during plant defense (Reverchon et al., 2002). Besides being present in most bacteria, INDA is found in the yeast *Candida elegans* and *Arabidopsis* (Finn et al., 2014). Based on the low expression level of this gene in *Arabidopsis* (Supplemental Figure S2), we predict that INDA may play a regulatory rather than housekeeping role and its expression may be under a tight temporal/spatial control. Among all the mutants tested in this study, plants lacking INDA exhibited the strongest phenotype across most assays. Although the biochemical pathway this protein is involved in remains speculative, INDA appears to have a profound effect on β-oxidation.

**B12D1 in β-oxidation**
The expression of the barley *B12D* gene (*HvB12Dg1*) is high in the aleurone layer and in the embryo of developing seeds, diminishes towards seed maturity, and reappears in germinating seeds (Aalen et al., 1994). Consistent with this, the expression of *HvB12Dg1* was shown to be regulated by two key hormones that control seed germination: up-regulation by gibberellic acid (GA3) and down-regulation by ABA (Steinum et al., 1998). Similarly, *Arabidopsis B12D1* is preferentially expressed during seed maturation and germination, causing poor seed germination in its absence. In cereal seeds, proteins localized in aleurone and embryo are involved in the synthesis and accumulation of lipid bodies, desiccation tolerance and dormancy (Aalen et al., 1994), yet the precise function of HvB12D1 is still unclear. We have shown that plants lacking B12D1 are resistant to 2,4-DB and accumulate oil bodies and long-chain fatty acids in germinated seedlings, suggesting B12D1’s involvement β-oxidation related processes during germination. The biochemical function of B12D1 remains to be determined.
Conclusions
Our systematic and quantitative analysis of mutants of recently discovered *Arabidopsis* peroxisomal genes identified additional peroxisomal proteins with varying degrees of contribution to β-oxidation. This study has taken a step forward towards completely dissecting the plant β-oxidation network, and provides a framework for future investigations to integrate genetics and physiology with biochemical and metabolic assays to identify substrates for all enzymes in β-oxidation.

MATERIALS AND METHODS

Plant material and growth conditions
*Arabidopsis thaliana* ecotype Columbia (Col-0 and Col-3) and Wassilewskija (Ws-4), and *pex14* (SALK_007441), *opcl1* (SALK_140659), *acx4-1* (SALK_000879) and *kat2-3* (SALK_024922) mutants were used as controls. Seed stocks were obtained from the *Arabidopsis* Biological Resource Center, the Nottingham *Arabidopsis* Stock Centre and the INRA-Versailles Genomic Resource Center (Table 1 and Supplemental Table S1). Surface sterilized seeds were plated on half-strength Linsmaier and Skoog (1/2 LS) media (Caisson Laboratories, Inc.) solidified with 0.8% agar (w/v) and supplemented with 1% (w/v) sucrose. Plants were grown at 22 °C under continuous illumination at 75 µmol photons m⁻² s⁻¹. Homozygous lines were identified by PCR analysis of genomic DNA from 7d seedlings. The location of the T-DNA insertion was confirmed by sequencing of the PCR products. Primers used are listed in Supplemental Table S5.

RNA analysis
For RT-PCR analysis of the mutants, total RNA was isolated from 7d seedlings grown on medium with 1% (w/v) sucrose as described previously (Mallory et al., 2001). After being treated with DNaseI (QIAGEN), 1 µg of each RNA sample was reverse transcribed using SuperScript® III Reverse Transcriptase kit (Invitrogen). The expression level of the target gene was analyzed by PCR using 50 ng cDNA for 25 (for *UBQ10*) or 35 (for target gene) cycles. The gene-specific primers used are summarized in Supplemental Table S5.

Physiological assays
For germination assays, seeds harvested from plants that had been grown simultaneously were sown on 0.8% agar (w/v) plates (i.e. plain agar plate) or 1/2 LS media solidified with 0.8% agar. The plates were kept at 4°C for 4 d before being transferred to growth chamber with continuous low light intensity (plates covered with mesh) or darkness. An additional set of plates was exposed to light for 1 h before being placed in the dark in growth chamber. After 5d, radicle emergence was scored. For seed germination in response to ABA, seeds were sown on plain agar plates supplemented with ABA (Sigma-Aldrich). After 4d stratification at 4 °C, plates were transferred to growth chamber and grown in the light for 5d or 10d before radicle emergence was scored. All the data are representative of at least three independent experiments. For each experiment, n=50.

For sucrose dependence analysis, seeds were placed on plates supplemented with or without 1% (w/v) sucrose and stratified at 4 °C for 2d. After 7d in growth chamber with continuous low intensity light or darkness, the plates were scanned using an EPSON scanner (Epson Perfection 4870 PHOTO). Hypocotyl lengths of dark-grown seedlings and roots of light-grown seedlings were measured using ImageJ (imagej.nih.gov/ij/). To study IAA, IBA, 2,4-D, 2,4-DB and OPDA responses, seeds were sown on plates containing 0.5% (w/v) sucrose and various concentrations of IAA (BioWORLD), IBA (Sigma-Aldrich), 2,4-D (Sigma-Aldrich), 2,4-DB (Sigma-Aldrich), OPDA (Cayman Chemical), or MeJA (Sigma-Aldrich), as described in figure legends. The plates were then stored at 4 °C for 2d and placed in growth chamber with continuous low-intensity light. Root length was quantified at 7d. All the data are representative of at least three independent experiments. For each experiment, n=50.

To detect photorespiratory phenotypes, 2w seedlings were transferred from plates to soil and placed in a growth cabinet with controlled environment at light intensity of 115 µmol photons m⁻² s⁻¹, 20 °C, 16/8 photoperiod, and CO₂ concentration of 80 µL L⁻¹ CO₂ (low CO₂ concentration) or 400 µL L⁻¹ CO₂ (ambient air). After two weeks, plants were photographed with a COOLPIX 8800 VR camera (Nikon).

**Visualization of oil bodies**
Etiolated seedlings grown on 0.8% agar (w/v) for 5 or 7d were stained for 5 min with 1 µg mL\(^{-1}\) aqueous solution of Nile Red (Molecular Probes) as previously described (Greenspan et al., 1985; Linka et al., 2008). Images were recorded using an Olympus FluoView 1000 Spectral-based Laser Scanning Confocal Microscope system (excitation wavelength 450-500 nm; emission wavelength, > 528 nm), using the 60x objective. All the data are representative of at least three independent experiments. For each experiment, n=12.

**Fatty acid analysis**

Amount of fatty acids in seeds and seedlings were analyzed as fatty acid methyl esters (FAMEs) according to a published protocol (Li et al., 2006). Briefly, 50 seedlings or 100 seeds were placed in a tube with a screw cap. Added to the tube were 2 mL of 5% (v/v) H\(_2\)SO\(_4\) in methanol, 300 µL of Toluene, and 10-60 µg of Tri15:0TAG (amount used depends on the tissue) as internal standard. The tubes were caped and vortexed to ensure submergence of the sample, and later heated for 90 min at 85 °C. The resulting FAMEs were extracted using hexane and 0.9% NaCl, dried down under nitrogen gas, and resuspended in appropriate volume of hexane. FAMEs were analyzed by gas chromatography (GC) with a flame ionization detector (FID). Samples were separated on a DB-23 capillary column (30 m x 0.25 mm ID, 0.25 µm film thickness; J&W Scientific, Folsom, CA), and helium was used as carrier gas at a constant flow of 1.5 mL/min. For GC, oven temperature was maintained at 140 °C for 3 min, followed by a 5 °C per min increase until the oven reached 230°C, and a final 3 min at 230°C. Injector and detector were maintained at 250 °C throughout the analysis.

**Statistical analysis**

To reveal statistical difference between the mutants and the wild type, all data generated from the assays were subjected to an unpaired Student’s \(t\) test. We considered differences between mutants and wild type plants with \(p<0.01\) as significant.

**SUPPLEMENTAL DATA**

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

**Supplemental Table S1.** Lines without T-DNA insertion and lines for which homozygotes could not be identified.
**Supplemental Table S2.** Identification of peroxisomal proteins analyzed in this study from previous proteomic studies.

**Supplemental Table S3.** Measurements for the sucrose dependence assays.

**Supplemental Table S4.** Log2 expression values downloaded from the BAR expression browser for heatmap generation.

**Supplemental Table S5.** Primers used for genotyping and RT-PCR analysis.

**Supplemental Figure S1.** Schematics of peroxisomal gens analyzed in this study.

**Supplemental Figure S2.** RT-PCR analysis of 7d mutant seedlings.

**Supplemental Figure S3.** Images of four-week-old plants grown in ambient air or under low CO₂.

**Supplemental Figure S4.** IAA and 2,4-D responses.

**Supplemental Figure S5.** IBA response of peroxisomal mutants.

**Supplemental Figure S6.** Responsiveness of Col-0 and pex14 to 2,4-DB.

**Supplemental Figure S7.** Responsiveness of mutants in Ws-4 background to 2,4-DB.

**Supplemental Figure S8.** Seed germination potential of peroxisomal mutants.

**Supplemental Figure S9.** Heatmap showing relative expression levels of candidate genes in seed maturation and germination.

**Supplemental Figure S10.** Visualization of hypocotyl oil bodies in 5d etiolated seedlings.

**Supplemental Figure S11.** Visualization of hypocotyl oil bodies in 7d etiolated seedlings.

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**AUTHOR CONTRIBUTIONS**
G.C. designed and performed experiments and data analysis. J.H. participated in the designing of experiments and data interpretation. G.C. and J.H. co-wrote the paper.

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FIGURE LEGENDS

Figure 1. Sucrose dependence assay.
Shown are hypocotyl lengths of 7d etiolated seedlings (A) and root lengths of 7d light-grown seedlings (B), both of which were grown on 1/2 LS medium with or without 1% Suc. Data represent means ± SE of three independent experiments. For each experiment, n≥30. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate statistically significant differences from the wild type (Col-0, Col-3 or Ws-4). Student’s t test, * p<0.01, ** p<0.001. Arrows denote mutants that show sugar dependence.

Figure 2. 2,4-DB response assay.
Percentage of 7d light-grown seedlings displaying resistance to 2,4-DB after growing on 1/2 LS medium supplemented with 0.5% Suc and 0.4, 0.8, or 1 μM of 2,4-DB is presented. Resistance
was defined as having root length with no statistically significant differences from that on medium without 2,4-DB. Data represent means ± SE of three independent experiments. For each experiment, n≥30. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate statistically significant changes from the wild type (Col-0, Col-3, or Ws-4). Student’s t test, * p<0.01, ** p<0.001.

Figure 3. Root response to OPDA.
Relative root lengths (treated vs. untreated) of 7d seedlings grown on 1/2 LS medium supplemented with 250 nM OPDA or 10 µM MeJA are shown. Data represent means ± SE of three independent experiments. For each experiment, n = 50. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate changes significantly different from that in the respective wild type (Col-0, Col-3 or Ws-4). Student’s t test, * p<0.01, ** p<0.001.

Figure 4. Seed germination in the mutants.
Percentage of radicle emergence from seeds on plain agar (0.8% agar) in dark without or with 1h light pre-treatment is presented. Data represent means ± SE of three independent experiments. For each experiment, n = 50. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate changes significantly different from that in the wild type (Col-0, Col-3, or Ws-4). Student’s t test, * p<0.01, ** p<0.001.

Figure 5. Seed germination in response to ABA.
Percentage of radicle emergence from seeds grown on plain agar supplemented with 0, 2 or 5 µM of ABA for 5 and 10d is presented. Data represent relative means (treated vs. untreated) ± SE of three independent experiments. For each experiment, n = 100. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate changes significantly different from that in the wild type (Col-0, Col-3 or Ws-4). Student’s t test, *p<0.001.

Figure 6. Confocal microscopic visualization of oil bodies in the hypocotyl of 7d etiolated seedlings.
Seedlings were grown on 0.8% agar plate. Nile red fluorescence images (top) and merged images (bottom) of bright-field and nile red fluorescence are shown. Scale bars = 10 µm. Area of fluorescence is the arbitrary value (assigned by Image J) within the 75-µm² area of nile red fluorescent images shown in the figure.

**Figure 7.** Fatty acid quantification in seedlings.
Percentage of fatty acids remaining is shown for 3d (A), 5d (B) and 7d (C) etiolated seedlings grown on 0.8% agar plates, as compared with the level in seeds. The amount of FA in dry seeds was set as 100%. Data represent means ± SE of three independent experiments. For each experiment, n≥50. Asterisks indicate changes significantly different from that in the wild type (Col-0, Col-3, or Ws-4). Student’s t test, * p<0.01, ** p<0.001.
Table 1: Confirmed homozygous peroxisomal mutants analyzed in this study. PTS, Peroxisomal targeting signal. Based on the level of the full-length target transcripts, mutants were considered to be KO, knock-out mutant; KD, knock-down mutant; OE: overexpressor mutant. β-oxidation assays: SC, sucrose dependence assay; DB, 2,4-DB assay; OPDA, OPDA assay; NR, detection of oil bodies using nile red; FA, fatty acids quantification; D/L, requires light for complete germination; ABA, seed germination in presence of ABA. a indicates mutants previously analyzed for their role in IBA and 2,4-DB β-oxidation processes; b indicates mutants previously characterized in processes other than β-oxidation. X, mutant exhibits a phenotype; -, mutant has no phenotype. N/A, not applicable.

| Gene Locus | Acronym | Annotation | PTS | Allele | Line | Ecotype | Level of target transcripts | β-oxidation processes |
|------------|---------|------------|-----|--------|------|---------|-----------------------------|----------------------|
| At1g01710  | ACH2    | Acyl-CoA thioesterase 2 | SKL | ach2-1 | SALK_134567 | Col0 | X | - | X | X | - | X | - | X |
|            |         |            |     | ach2-2 | GKL_705E01  | Col0 | X | - | X | X | - | X | - | X |
|            |         |            |     | ach2-3 | SALK_126817C | Col0 | X | - | X | X | - | X | - | X |
| At1g16730  | UP6     | Unknown protein 6 | CRL | up6-1  | SALK_122395 | Col0 | X | - | X | X | - | N/A | X | X |
| At1g19570  | DHAR1   | Dehydroascorbate reductase 1 | DRL | dhar1-1 | SALK_005382 | Col0 | X | - | X | - | - | N/A | X | X |
|            |         |            |     | dhar1-2 | SALK_029966C | Col0 | X | - | X | - | - | N/A | X | X |
| At1g20560  | AAE1    | Acyl-activating enzyme isoform 1 | SKL | aae1-1 | SALK_041152 | Col0 | - | - | - | X | - | N/A | X | X |
| At1g48320  | DHNAT1/  | DHNA-CoA thioesterase 1/ Small thioesterase | AKL | dhnat1-1 | CS846230  | Col0 | X | - | X | X | - | N/A | X | X |
| sT1        |         |            |     | dhnat1-2 | SALK_066477C | Col0 | X | - | - | - | - | N/A | X | X |
| At1g50510  | INDA    | Indigoidine synthase A | RTxHL | inda-1 | GKL_61801 | Col0 | X | X | - | - | X | X | X | X |
| At2g29590  | sT5     | Small thioesterase 5 | SML | st5-1  | SALK_095212 | Col0 | X | - | - | X | - | N/A | X | X |
|            |         |            |     | st5-2  | SALK_092512 | Col0 | X | - | - | X | - | N/A | X | X |
| At2g42490  | CuAO3   | Copper amine oxidase 3 | SKL | cuo3-1 | SALK_095214 | Col0 | X | - | - | X | - | N/A | X | X |
|            |         |            |     | cuo3-2 | SALK_096063C | Col0 | X | - | - | - | - | N/A | X | X |
| At3g12800  | SDRb /  | Short-chain dehydrogenase/ reductase | SKL | decc-2 | CS877641  | Col0 | X | - | - | - | - | N/A | X | X |
| DECR       |         | isoform b/ 2,4-dienoyl-CoA reductase |     | decc-3 | CS871159  | Col0 | X | - | - | - | - | N/A | X | X |
| At3g14150  | HAOX2   | Hydroxy-acid oxidase isoform 2 | SML | haox2-1 | SALK_102409C | Col0 | X | - | - | - | - | N/A | N/A | N/A |
| At3g18140  | B12D1   | Senescence-associated protein/ B12D-related protein | SML | b12d1-1 | FLAG_548C12 | Col0 | X | - | - | - | - | N/A | N/A | N/A |
| At3g18170  | BADH    | Betaine Aldehyde dehydrogenase | SKL | badh-1 | CS822971  | Col0 | X | - | - | X | - | N/A | X | X |
| At3g55640  | CADC    | Ca2⁺-dependent carrier | SKL | cadc-1 | CS832927  | Col0 | X | - | - | - | - | N/A | X | X |
| At3g56460  | ZnDH    | Zinc-binding dehydrogenase | SKL | zndh-1 | SALK_056640 | Col0 | X | - | - | - | - | N/A | N/A | N/A |
| Accession | Symbol | Description                                      | Source | Allele 1 | Allele 2 | Allele 3 | Allele 4 | Allele 5 | Allele 6 | Allele 7 | Allele 8 | Allele 9 | Allele 10 | Allele 11 | Allele 12 | Allele 13 | Allele 14 | Allele 15 |
|-----------|--------|-------------------------------------------------|--------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| At4g02340 | EH3    | Epoxide hydrolase isoform 3                      | ASL    | eh3-1    | eh3-2    | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      |
| At4g04320 | MCD    | Malonyl-CoA decarboxylase                        | SRL    | mod-1    | mod-2    | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      |
| At4g09320 | NDPK1  | Nucleoside diphosphate kinase type 1             | SRL    | ndpk1-1  | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      |
| At5g04870 | CPK1   | Calcium dependent protein kinase 1              | SRL    | cpk1-1b  | cpk1-2b  | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      |
| At5g11910 | ELT1   | Esterase/ lipase/ thioesterase family isoform 1 | SRI    | elt1-1   | FLAG_632F03 | Ws4     | X        | X        | N/A      | N/A      | N/A      | N/A      | N/A      | N/A      | X        | X        | X        | X        |
| At5g16370 | AAE5   | Acyl-activating enzyme 5                         | SRM    | aae5-1*  | SALK_009731 | Col0    | X        | -        | X        | X        | X        | X        | X        | X        | X        | X        | X        | X        |
| At5g17720 | ACAT1.3/AAC1T | Acetoacetyl-CoA thiolase 1.3 | SAL | aact1-2* | SALK_008505 | Col0 | X | - | - | - | X | X | X | X | X |
| At5g18820 | TTL1   | Transthyretin-like protein 1                     | RLxHL  | ttl-3    | SALK_137289 | Col0 | X | - | - | - | N/A | N/A | N/A | N/A | N/A | N/A |
| At5g58400 | UP7    | Serine Hydrolase 1                               | SLM    | up7-1    | SALK_062218C | Col0 | X | X | - | - | N/A | N/A | X | X | X | X | X |
Figure 1. Sucrose dependence assay. Shown are hypocotyl lengths of 7d etiolated seedlings (A) and root lengths of 7d light-grown seedlings (B), both of which were grown on LS medium with or without 1% Suc. Data represent means ± se of three independent experiments. For each experiment, n≥30. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate statistically significant differences from the wild type (Col-0, Col-3 or Ws-4). Student’s t test, * p<0.01, ** p<0.001. Arrows denote mutants that show sugar dependence.
Figure 2. 2,4-DB response assay.
Percentage of 7d light-grown seedlings displaying resistance to 2,4-DB after growing on 1/2 LS medium supplemented with 0.5% Suc and 0.4, 0.8, or 1 µM of 2,4-DB is presented. Resistance was defined as having root length with no statistically significant differences from that on medium without 2,4-DB. Data represent means ± se of three independent experiments. For each experiment, n≥30. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate statistically significant changes from the wild type (Col-0, Col-3, or Ws-4). Student’s t test, * p<0.01, ** p<0.001.
Figure 3. Root response to OPDA.
Relative root lengths (treated versus untreated) of 7d seedlings grown on 1/2 LS medium supplemented with 250 nM OPDA or 10 µM MeJA are shown. Data represent means ± se of three independent experiments. For each experiment, n = 50. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate changes significantly different from that in the respective wild type (Col-0, Col-3 or Ws-4). Student’s t test, *p<0.01, **p<0.001.
Figure 4. Seed germination in the mutants.
Percentage of radicle emergence from seeds on plain agar (0.8% agar) in dark without or with 1h light pre-treatment is presented. Data represent means ± se of three independent experiments. For each experiment, n = 50. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate changes significantly different from that in the wild type (Col-0, Col-3, or Ws-4). Student’s t test, * p<0.01, ** p<0.001).
Figure 5. Seed germination in response to ABA. Percentage of radicle emergence from seeds growing on plain agar supplemented with 0, 2 or 5 µM of ABA for 5 and 10d is presented. Data represent relative means (treated vs untreated) ± se of three independent experiments. For each experiment, n = 100. Section and hashtag signs indicate mutants in Col-3 and Ws-4 backgrounds respectively. Asterisks indicate changes significantly different from that in the wild type (Col-0, Col-3 or Ws-4). Student’s t test, *p<0.001.
Figure 6. Confocal microscopic visualization of oil bodies in the hypocotyl of 7d etiolated seedlings. Seedlings were grown on 0.8% agar plate. Nile red fluorescence (top) and merged images (bottom) of bright-field and nile red fluorescence are shown. Scale bars = 10 µm. Area of fluorescence is the arbitrary value (assigned by Image J) within the 75-µm² area of nile red fluorescent images shown in the figure.
Figure 7. Fatty acid quantification in seedlings. Percentage of fatty acids remaining is shown for 3d (A), 5d (B) and 7d (C) etiolated seedlings grown on 0.8% agar plates, as compared with the level in seeds. The amount of FA in dry seeds was set as 100%. Data represent means ± SE of three independent experiments. For each experiment, n ≥ 50. Asterisks indicate changes significantly different from that in the wild type (Col-0, Col-3, or Ws-4). Student’s t test, * p<0.01, ** p<0.001.