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Cytochrome b5 Reductase Encoded by CBR1 Is Essential for a Functional Male Gametophyte in Arabidopsis

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In all eukaryotes, NADH:cytochrome b5 reductase provides electrons, via cytochrome b5, for a range of biochemical reactions in cellular metabolism, including for fatty acid desaturation in the endoplasmic reticulum. Studies in mammals, yeast, and in vitro plant systems have shown that cytochrome b5 can, at least in some circumstances, also accept electrons from NADPH:cytochrome P450 reductases, potentially allowing for redundancy in reductase function. Here, we report characterization of three T-DNA insertional mutants of the gene encoding cytochrome b5 reductase in Arabidopsis thaliana, CBR1. The progeny of plants heterozygous for the cbr1-2 allele segregated 6% homozygous mutants, while cbr1-3 and cbr1-4 heterozygotes segregated 1:1 heterozygous: wild type, indicating a gametophyte defect. Homozygous cbr1-2 seeds were deformed and required Suc for successful germination and seedling establishment. Vegetative growth of cbr1-2 plants was relatively normal, and they produced abundant flowers, but very few seeds. The pollen produced in cbr1-2 anthers was viable, but when germinated on cbr1-2 or wild-type stigmas, most of the resulting pollen tubes did not extend into the transmitting tract, resulting in a very low frequency of fertilization. These results indicate that cytochrome b5 reductase is not essential during vegetative growth but is required for correct pollen function and seed maturation.

INTRODUCTION

The transfer of electrons is fundamental to metabolism within the cell. Distinct from the chloroplast and mitochondrial redox pathways, there are two prominent electron transfer systems in the endoplasmic reticulum (ER) membrane. One operates through NADH:cytochrome P450 reductase, transferring electrons to the many cytochrome P450 monoxygenases, and the other operates through NADPH:cytochrome b5 reductase, transferring electrons to cytochrome b5 proteins. In plants, a primary role for the cytochrome b5 electron transfer system is to supply reductant for the synthesis of the polyunsaturated fatty acids linoleic acid (18:2) and α-linolenic acid (18:3) (Slack et al., 1976; Kearns et al., 1991; Shankan and Cahoon, 1998), which are integral to the function of cellular membranes (McConn and Browse, 1998). Synthesis of polyunsaturated fatty acids occurs in all plant tissues, and in developing seeds of Arabidopsis thaliana, they make up half of triacylglycerol, the storage lipid that is the principal component of seed oil. Human consumption of triacylglycerol extracted from oilseeds provides 18:2 and 18:3 fatty acids that are both essential dietary components and precursors for the synthesis of 20- and 22-carbon polyunsaturated fatty acids required for human health (Wallis et al., 2002). In the ER of plant cells, FATTY ACID DESATURASE2 (FAD2) converts oleic acid (18:1) to 18:2 (Miquel and Browse, 1992; Okuley et al., 1994), followed by conversion of 18:2 to 18:3 by the FAD3 linoleate desaturase (Browse et al., 1993). Mammalian cytochrome b5 reductase (EC 1.6.2.2) is an ER flavoprotein that transfers electrons from NADH to the FAD prosthetic group of the reductase and subsequently to the heme of cytochrome b5 (Percy and Lappin, 2008). The crystal structure shows two cytoplasmic domains, a FAD binding domain and a NADH binding domain, joined by a linker region and a single transmembrane region at the N terminus (Bando et al., 2004; Bewley et al., 2001). These structural components are strongly conserved in the family of NADH:cytochrome b5 reductases, including CBR1 of Arabidopsis. The links that follow CBR1 in electron transport are the five Arabidopsis cytochrome b5 proteins (CB5-A to CB5-E) and one CB5-like protein (Nagano et al., 2009); four of these are predicted to localize to the ER membrane (Hwang et al., 2004; Nagano et al., 2009). Although the specific functions of individual CB5 proteins are unknown, there is likely to be some degree of redundancy. Several enzymes also contain cytochrome b5 domains (Sperling et al., 1998). A second Arabidopsis cytochrome b5 reductase, CBR2, is localized to the inner mitochondrial membrane, and there is no evidence that it is part of the ER electron transport system (Heazlewood et al., 2004).

Whereas fatty acid desaturation is a major function of electron transfer through cytochrome b5, additional enzymes of primary and secondary metabolism also rely on cytochrome b5 to provide electrons. These include enzymes contributing to the synthesis and modification of cutin, suberin, cuticular waxes, sterols, and sphingolipids (Boutte and Grebe, 2009; Li-Beisson et al., 2013;
CBR1 and Male Gametophyte Function

Markham, et al., 2013). In sphingolipid metabolism, there are five cytochrome b5-dependent hydroxylation and desaturation reactions that contribute to the synthesis of the predominant glycosyl inositolphosphoceramides and glucosylceramides that are important components of the plasma membranes in plant cells (Markham, et al., 2013).

CBR1-mediated electron transfer through cytochrome b5 to fatty acid desaturases may not be the only electron sources for some of these enzymes. Reports indicate that NADPH:cytochrome P450 reductase, encoded by ARABIDOPSIS THALIANA REDUCTASE1 (ATR1) and ATR2 in Arabidopsis, can transfer electrons from NADPH to cytochrome b5; these electrons would be available to enzymes of the cytochrome b5 pathway (Fukuchi-Mizutani et al., 1999). In mammals, there is often functional overlap between CBR1 and NADPH:cytochrome P450 reductase, for example, when NADPH-dependent reductase proteins supply electrons to cytochrome b5 in microsomal preparations (Enoch and Strittmatter, 1979; Dür et al., 2007). Genetic experiments demonstrate nearly complete redundancy of the two electron transfer systems in yeast (Saccharomyces cerevisiae). Yeast with null mutations in either Cbr1 or Ncp1 (YHR042W, the yeast ATR homolog) are viable, with somewhat slower growth, whereas the double null mutations are lethal (Tiedje et al., 2007). Strong in vivo evidence for redundancy in electron transfer systems comes from the effects of a point mutation in Arabidopsis CBR1 that severely reduces its activity but only produces a decrease in 18:3 in seed fatty acid; aside from that effect, the mutant plants are indistinguishable from the wild type in growth, development, and seed production (Kumar et al., 2006). This implies that, apart from a fairly subtle change in oil formation, CBR1 is not critical to plant health. Since in many cases electrons can be supplied to cytochrome b5 proteins and P450 enzymes either from NADH, via cytochrome b5 reductase, or from NADPH, via P450 reductase, it seems likely that the P450 reductase could support normal Arabidopsis growth in the absence of CBR1, similar to the phenotype seen in yeast.

In contrast with the hypothesis that CBR1 is dispensable, we report here that three CBR1 T-DNA insertion knockout lines suffered distorted segregation, with greatly reduced penetrance through the male gametophyte. It was possible to identify plants homozygous for only one of the three mutations, cbr1-2, and for that line only 6% of the progeny were homozygous when a heterozygote was allowed to self-pollinate. Homozygous cbr1-2 plants produced very few seeds, and these exhibited reduced 18:3, decreased total oil content, and delayed germination. In fertilization experiments, pollen of cbr1-2 plants germinated on wild-type and cbr1-2 stigmas, but the majority of pollen tubes stopped growing prior to reaching the ovules, leading to severely reduced fertilization and seed set. Our work reveals that, in Arabidopsis, CBR1 is required for pollen tube growth and normal fertilization.

RESULTS

Identification of Three T-DNA Insertion Alleles in CBR1

The ethyl methanesulfonate–generated mutant cbr1-1 has decreased levels of 18:3 due to instability of the mutated CBR1 protein and reduced enzymatic activity but is otherwise like wild-type Arabidopsis (Kumar et al., 2006). To characterize the effects of complete loss of function in CBR1, we obtained several T-DNA insertional alleles of cbr1 from the ABRC and Gabi-Kat (Rosso et al., 2003). After preliminary analysis (see Methods for details), three alleles were selected for further characterization. PCR analysis of Sail_644_A11, renamed cbr1-2, revealed a tandem insertion located in the middle of exon three, with two left borders facing genomic DNA; 28 nucleotides of genomic DNA are unaccounted for between the two left borders (Figure 1A). The left border of this insertion that is oriented toward the 3' end of the gene is described correctly in the SIGnAL T-DNA Express database (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al., 2003). Our sequence analysis shows that the WiscDslox 377-380021 insertion (Woody et al., 2007), renamed cbr1-3, is located in the second to last nucleotide at the end of exon six (Figure 1A), not in the following intron as described in the SIGnAL database. Finally, the Gabi-Kat 371A08 insertion, renamed cbr1-4, is a tandem insertion with two left borders facing genomic DNA, one left border in exon two and the other in intron one; 188 nucleotides of genomic DNA are unaccounted for between the two left borders (Figure 1A).

cbr1 Mutants Have Distorted Segregation

These three T-DNA lines, obtained from the stock centers as segregating T2 seeds, were germinated on agar medium and transferred to soil; cbr1-2 and cbr1-3 plants were treated with Basta to select for the T-DNA insertions. Plants of all three lines were genotyped by PCR in an attempt to identify homozygous individuals. In the T2 generation, we found that cbr1-2, cbr1-3, and cbr1-4 did not segregate homozygous plants (Table 1), so heterozygous plants from each line were allowed to self-pollinate and their progeny were genotyped. For the cbr1-2 mutant, of 106 plants screened, only six homozygous plants were identified in the T3 generation, representing 6% of the total (Table 1, Figure 1B), significantly lower than the 25% predicted for Mendelian segregation. In this segregating T3 population of cbr1-2, 49% of the plants were heterozygous for the cbr1-2 insert and 45% were wild type (Table 1). When the T3 progeny of heterozygous cbr1-3 or cbr1-4 plants were analyzed, only 46% of the cbr1-3 and 49% of the cbr1-4 carried the T-DNA insertion; no line homozygous for either of these two insertions could be obtained (Table 1). The cbr1-3 and cbr1-4 mutant lines continued to segregate in this fashion in the T4 generation; no homozygous plants from either allele were identified (Figure 1B, Table 1), even when more than 500 progeny from cbr1-3 heterozygotes were screened. Because the initial segregating cbr1-4 T2 seedlings exhibited 98% resistance to sulfadiazine (the selectable marker of the T-DNA), indicating multiple T-DNA insertions, a backcross to wild-type Arabidopsis was necessary (described in Methods). From the backcross, a heterozygous cbr1-4 plant was isolated in which 50% of the self-pollinated progeny were resistant to sulfadiazine, but again no homozygous plant was detected. The distorted segregation observed in all of these T-DNA lines, with approximately a 1:1 segregation of heterozygous to the wild type, suggests that absence of CBR1 results in a gametophytic defect.
The full siliques observed on heterozygous cbr1 mutant plants suggested that the female gametophyte was functional, but to define which gametophyte was affected in these mutants, we reciprocally backcrossed each of the three cbr1 heterozygous mutants to wild-type Arabidopsis (Columbia-0) plants, with the expectation that segregation would occur in a Mendelian ratio of 1:1 (heterozygous:wild type). When each of the heterozygous mutant lines was fertilized with the pollen of wild-type plants (CBR1/cbr1) this expectation was met: cbr1 female gametophytes were functional. However, for the reciprocal crosses using heterozygous mutant plants as the pollen donor (wild type × CBR1/cbr1), the cbr1 allele was transmitted at very low frequency.

**Table 1. Segregation Analysis of the cbr1 Mutant Alleles in Progeny From Self-Pollinated cbr1 Heterozygotes**

<table>
<thead>
<tr>
<th>Genotype from PCR Analysis</th>
<th>% Carrying T-DNA</th>
<th>cbr1/cbr1</th>
<th>CBR1/cbr1</th>
<th>CBR1/CBR1</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBR1/cbr1-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2 plants</td>
<td>43%</td>
<td>0</td>
<td>22</td>
<td>29</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T3 plants</td>
<td>55%</td>
<td>6</td>
<td>52</td>
<td>48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CBR1/cbr1-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2 plants</td>
<td>62%</td>
<td>0</td>
<td>18</td>
<td>11</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T3 plants</td>
<td>42%</td>
<td>0</td>
<td>45</td>
<td>62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T4 plants</td>
<td>57%</td>
<td>0</td>
<td>20</td>
<td>15</td>
<td>0.0011</td>
</tr>
<tr>
<td>CBR1/cbr1-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2 plants</td>
<td>63%</td>
<td>0</td>
<td>19</td>
<td>11</td>
<td>0.0061</td>
</tr>
<tr>
<td>T3 plants</td>
<td>49%</td>
<td>0</td>
<td>19</td>
<td>20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T4 plants</td>
<td>53%</td>
<td>0</td>
<td>16</td>
<td>14</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

Heterozygous cbr1 plants were allowed to self-pollinate for each generation. The observed segregation ratios of the progeny were compared with the expected Mendelian ratio of 1:2:1; P values were calculated using the χ² test, 2 df.

*Significant differences between the observed ratio and the expected ratio 1:2:1.
low rates. Most of the progeny were wild type in these test crosses: 24 of 24 were wild type for the \textit{CBR1/cbr1-2} testcross, 26 of 28 for the \textit{CBR1/cbr1-3} testcross, and 15 of 18 were wild type for the \textit{CBR1/cbr1-4} testcross. The low transmission of all three \textit{cbr-1} alleles indicates that the male gametophytes of the \textit{cbr1} mutants are substantially nonfunctional. We later genotyped, by PCR, 283 F2 progeny of two self-pollinated heterozygous plants derived from the wild type $\times$ \textit{CBR1/cbr1-3} testcross and found no homozygotes. Similar results were observed for the wild type $\times$ \textit{CBR1/cbr1-4} testcross, with no homozygous mutants identified among progeny of heterozygotes. Because there was some transmission of \textit{cbr1} alleles through the pollen, the testcross results indicate that a small proportion of \textit{cbr1} pollen is functional, and we infer that zygotes homozygous for \textit{cbr1-3} and \textit{cbr1-4} insertion alleles did not develop into viable embryos. Even though our test-crosses to the wild type indicated low viability of \textit{cbr1-2} pollen, homozygous \textit{cbr1-2} mutants were identified as viable plants.

**Initial Characterization of the Homozygous \textit{cbr1-2} Mutant**

The viable \textit{cbr1-2} homozygotes obtained allowed us to carry out phenotypic and biochemical analyses. First, homozygous \textit{cbr1-2} plants were analyzed for the presence of \textit{CBR1} transcript. Despite extensive RT-PCR analysis, no full-length transcript was identified from homozygous \textit{cbr1-2} (Figure 1C), even after a secondary nested PCR of the RT-PCR product (see Supplemental Figure 1A online). Additional RT-PCR using primers specific to the region of \textit{cbr1-2} 5' to the T-DNA insertion site also failed to detect any RNA (see Supplemental Figure 1B online). However, RT-PCR produced a product corresponding to \textit{CBR1} sequence 3' of the T-DNA insertion (Figure 1C). To determine if part of the T-DNA insert was transcribed with this \textit{CBR1} mRNA, we designed primers (see Supplemental Table 1 online) to amplify from within the T-DNA left border to the 3' end of \textit{CBR1}. RT-PCR indicated that amplification was possible more than 1300 bp into the T-DNA insertion (see Supplemental Figure 1B online). We consider it unlikely that this artifactual transcript could be translated into a functional protein. The transcript has stop codons in all three reading frames, including one in frame with the \textit{CBR1} coding sequence 105 bp upstream of the first preserved codon in the \textit{CBR1} open reading frame. The \textit{cbr1-2} insertions disrupt the \textit{CBR1} reading frame at amino acid residue 92, and based upon the three-dimensional crystal structure of human \textit{CBR1}, the mutation removes four segments of the six-region Greek-key $\beta$-barrel that forms the FAD binding domain (Bando et al., 2004). Mutation in this critical domain most likely eliminates or severely compromises activity of any protein produced.

Mature \textit{cbr1-2} seeds were shrunken and shriveled compared with wild-type seeds (Figure 2A), and quantitative measurements confirmed their deficiencies. Both average seed weight (Figure 2B) and oil per seed, measured as total fatty acid methyl esters (Figure 2C), were significantly less than the wild type. In sum, oil per seed weight was reduced from $359 \pm 13 \mu$g $\text{mg}^{-1}$ in the wild type to $281 \pm 20 \mu$g $\text{mg}^{-1}$ for \textit{cbr1-2} seeds. To investigate the possible developmental basis of the morphologically distorted seeds, we examined embryo development from 2 to 7 d after flowering using differential interference

![Figure 2. Characterization of Homozygous \textit{cbr1-2} Mature Seeds.](A) Mature seeds of the wild type (WT) (left) and \textit{cbr1-2} (right). (B) Average seed weight. (C) Total fatty acid methyl esters (FAME) per seed. (D) Fatty acid composition of mature seeds of the wild type and \textit{cbr1-2}. Data are mean $\pm$ SE for 18 replicates of 20 seeds each for (B) and (C) and mean $\pm$ SE for three replicates for (D). [See online article for color version of this figure.]
contrast microscopy. Up to the late torpedo stage of embryo development, we observed no visible differences between wild-type and cbr1-2 embryos (Figures 3A to 3F). However, while wild-type embryos consistently proceeded to the folded-cotyledon stage (Figure 3G), the development of cbr1-2 embryos was very variable. Some embryos folded like the wild type, as shown in Figure 3H, while others folded incompletely (Figure 3I) or did not fold (Figure 3J). These observations are consistent with the morphology of cbr1-2 seeds shown in Figure 2A and indicate that the deficiency in cbr1-2 plants affects the late stages of embryo and seed development.

In cbr1-2 seed, the proportion of 18:3 was significantly reduced. For wild-type Arabidopsis seed, 18:3 is an abundant fatty acid, making up 21% of the total fatty acids, but cbr1-2 seed had only 6% 18:3 (Figure 2D), and there was a concomitant increase in 18:1 in the mutant. Calculations from the data in Figure 2D indicate that 18:1 desaturation was reduced by 25% and 18:2 desaturation by 62% in cbr1-2 seeds, relative to wild-type controls. These changes are only slightly greater in magnitude than those seen in the hypomorphic mutant cbr1-1 (Kumar et al., 2006) and suggest a differential effect of reduced electron supply on activities of the FAD2 and FAD3 enzymes. Analyses of individual phospholipids from seeds also showed decreases in 18:3 (see Supplemental Figure 2 online), reflecting the fact that phosphatidylcholine is the substrate for fatty acid desaturation. Changes in fatty acid composition were confined to the seeds; there was no substantial change in fatty acid composition in the leaves or roots of cbr1-2 plants (see Supplemental Table 2 online).

Homozygous cbr1-2 seeds germinated poorly on soil and the resulting seedlings did not establish and grow into mature plants. As shown in Figure 4A, on agar medium in the absence of Suc, some cbr1-2 seeds germinated (scored by radicle protrusion), but did not produce a radicle greater than 1 mm in length even after 8 d, at which time ~90% of the wild-type controls were established seedlings with substantial roots. When seeds were sown on agar medium supplemented with 1% Suc, ~60% of cbr1-2 seeds germinated, compared with 98% of wild-type seeds. All of the germinated cbr1-2 seeds produced a radicle >3 mm in length and were scored as established seedlings (Figure 4A). Seeds of the cbr1-2 mutant also exhibited delayed germination, with the median time to germinate on sucrose medium >1.5 d compared with 0.6 d for wild-type controls (Figure 4B). The success of seedling establishment was highly variable, even after an extended, 7-d stratification regime. As shown in Figure 5A, compared with wild-type controls, cbr1-2 seedlings were delayed in root and leaf development after 7 d of growth. Nevertheless, after 10 d of growth on Suc medium, ~55% of cbr1-2 seedlings had expanded cotyledons and developing leaves. When these established seedlings were transferred to soil, the cbr1-2 plants varied in size, with most having smaller leaves and rosettes than wild-type controls (Figures 5B and 5C). The small proportion of cbr1-2 that germinated quickly and developed in synchrony with wild-type seedlings grew into plants whose appearance and height were close to the wild type (Figure 5D), indicating that much of the size variation observed in cbr1-2 plants was due to delayed germination and poor seedling establishment. To test whether the cbr1-2 mutation caused a detectable change in desaturation or hydroxylation of any individual glycerolipid or sphingolipid, we conducted lipidomics analyses on wild-type and cbr1-2 leaf extracts. No statistically significant difference in composition was detected in any of the 13 glycerolipid and sphingolipid components analyzed, and the quantities of these individual lipids in cbr1-2 were entirely comparable to those in the wild type (see Supplemental Figures 3 and 4 online).

**Complementation of cbr1 Mutants**

To confirm that the phenotypic defects observed in cbr1 mutant plants are the result of a cytochrome b5 reductase defect, we cloned a 3-kb genomic fragment containing the entire wild-type CBR1 gene into a plant expression vector. Transformation of

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**Figure 3.** Embryo Development in Wild-Type and cbr1-2 Seeds.

(A) to (F) From the heart to late-torpedo stage, wild-type (top row) and cbr1-2 (bottom row) embryos are similar in appearance. (G) to (J) The folded cotyledons visible in wild-type embryos (G) are seen in only some cbr1-2 embryos (H); other cbr1-2 embryos fold poorly (I) or not at all (J).
homozygous cbr1-2 plants with this CBR1 genomic clone restored both 18:3 and 18:1 fatty acids in T2 seed to wild-type levels (see Supplemental Table 3 online). Germination and seedling growth of the resulting cbr1-2 CBR1 T2 transgenic plants were also restored by the complementation transgene and were similar to the wild type. Almost all of these plants were also restored by the complementation transgene cbr1-2 CBR1 seedling growth of the resulting plants.

**Figure 4.** Germination and Establishment of cbr1-2.

(A) Germination (radicle protrusion) and establishment for wild-type (WT) or homozygous cbr1-2 seeds on agar media with or without 1% Suc. Establishment was scored as root length >3 mm at 8 d. Data are mean ± se for two experiments with 70 to 80 seeds per treatment. (B) Time course of germination (radicle protrusion) on agar media with 1% Suc. Data are mean ± se for four experiments with 20 to 35 seeds per replicate.

Homozygous cbr1-2 plants with this CBR1 genomic clone restored both 18:3 and 18:1 fatty acids in T2 seed to wild-type levels (see Supplemental Table 3 online). Germination and seedling growth of the resulting cbr1-2 CBR1 T2 transgenic plants were also restored by the complementation transgene and were similar to the wild type. Almost all of these cbr1-2 plants contained the complementation transgene, since transmission of the cbr1-2 allele was very low in noncomplemented pollen and noncomplemented homozygous cbr1-2 seeds germinated at a low rate. This complementation was equally successful for the other insertion alleles. When plants heterozygous for the cbr1-3 mutation were transformed with the CBR1 genomic clone, it was possible using PCR to identify, among the self-pollinated progeny of the transformants, plants homozygous for the cbr1-3 mutation, which never occurred in the untransformed mutant line. All homozygous plants contained the CBR1 transgene, and no homozygous cbr1-3 plants were identified that lacked the CBR1 transgene (see Supplemental Table 4 online). To complement the third insertion mutant, heterozygous cbr1-4 plants were crossed with a complemented cbr1-3/CBR1 line. As observed for the other mutants, cbr1-4 homozygotes were obtained when, and only when, the line contained the CBR1 transgene (see Supplemental Table 4 online). Complementation of all three cbr1 insertion mutants by the CBR1 genomic clone demonstrates that the phenotypes described result from mutations within the CBR1 gene.
tract and reached an ovule, a low success rate of pollen tube growth that correlates well with the observed seed set of cbr1-2 plants (Figure 6). In germination tests on artificial media (Boavida and McCormick, 2007), only 3% of cbr1-2 pollen grains produced a pollen tube >20 μm long. Therefore, attenuated pollen tube growth in cbr1-2 is principally responsible for low seed production in the mutant line.

To confirm that the CBR1 gene was actively transcribed in pollen and pollen tubes, we fused the CBR1 promoter to the β-glucuronidase (GUS) reporter (Curtis and Grossniklaus, 2003) and transformed wild-type Arabidopsis plants with the construct. When pollen from T1 plants was germinated in vitro (Boavida and McCormick, 2007) and stained for GUS activity, it was readily apparent that the CBR1 promoter was active in both hydrated pollen and growing pollen tubes (Figure 8C), consistent with results from transcriptional profiling experiments (Wang et al., 2008).

Pollen tube guidance and fertilization can be disrupted by mutations affecting sporophytic tissues, such as the transmitting tract or embryo sac, or by the female gametophyte, so even though our testcrosses indicate a defective male gametophyte, the results do not preclude an additional contribution from the maternal tissue or female gametophyte. To test this possibility, we conducted reciprocal testcrosses between the wild type and cbr1-2 and stained the pistils 24 h after pollination with aniline blue to assess pollen tube growth. When wild-type pollen was used as the pollen donor for either wild-type pistils (wild type × cbr1-2) or mutant pistils (cbr1-2 × wild type), pollen tube growth was prolific throughout the transmitting tract (Figure 9A). By contrast, when cbr1-2 pollen was used as the pollen donor for either mutant (cbr1-2 × cbr1-2) or wild-type pistils (wild type × cbr1-2), pollen apparently germinated but did not extend normal pollen tubes (Figure 9A). When assessed at 9 d after pollination, wild type × cbr1-2 seed set (Figures 9B and 9C) was similar to the seed set from cbr1-2 self-pollinated siliques (Figure 6). These results show that the dysfunctional pollen tube growth of cbr1-2 is substantially independent of the female gametophyte and that the critical requirement for CBR1 is in pollen tube growth.

DISCUSSION

Biochemical Compensation for the Loss of CBR1

Cytochrome b5 reductase transfers electrons from NADH to cytochrome b5 proteins, which then transfer the electrons to fatty acid desaturases (Shanklin and Cahoon, 1998), hydroxylases (Nagano et al., 2009), and other enzymes. While not yet characterized biochemically, enzymes with N-terminal cytochrome b5 domains that incorporate a characteristic heme binding motif most likely acquire electrons via CBR1 and cytochrome b5, as has been established in the family of desaturases with similar cytochrome b5 domains (Sperling et al., 1998; Gostincar et al., 2010).

There is considerable evidence that NADPH:cytochrome P450 reductase, whose role is to transfer electrons from NADPH to the cytochrome P450 monoxygenases, can also supply electrons to the cytochrome b5 pathway. In vitro analysis of mammalian electron transfer demonstrates that the P450 reductase can supply electrons to cytochrome b5 for fatty acid desaturation (Enoch and Strittmatter, 1979; Dailey and Strittmatter, 1980). In addition, P450 reductase supports or enhances many
cytochrome b5 activities (Guengerich, 2005). Mice deleted in cytochrome b5 are viable, but greatly perturbed in their P450 metabolism, demonstrating in vivo the interconnection between the two electron transfer pathways (Finn et al., 2011). Indeed, in *S. cerevisiae*, cytochrome b5 reductase activity is completely dispensable, while simultaneous loss of cytochrome b5 reductase and P450 reductase is lethal (Tiedje et al., 2007).

Compensation for loss of cytochrome b5 reductase is not always so complete. Human methemoglobinemias are genetic diseases caused by mutations affecting cytochrome b5 electron transfer. In milder forms of the disease, these mutations produce treatable cyanosis (Hultquist et al., 1993), but in severe cases disruption in fatty acid and cholesterol metabolism damages neurological development, resulting in death during early childhood (Percy et al., 2005). Clearly, NADPH:cytochrome P450 reductase is incapable of fulfilling all roles of CBR1 in humans.

In plants, recombinant *Arabidopsis* P450 reductase proteins have been shown to supply electrons to cytochrome b5 proteins in vitro (Fukuchi-Mizutani et al., 1999), suggesting at minimum that support for cytochrome b5 activity is possible through the P450 reductase electron transfer pathway. An *Arabidopsis* mutant with greatly reduced cytochrome b5 reductase activity exhibited no phenotypic change other than reduction of 18:3 fatty acids in mature seeds (Kumar et al., 2006), demonstrating that much of the cytochrome b5 reductase activity is dispensable. However, our examination of *cbr* insertional mutants shows that loss of cytochrome b5 reductase disrupts reproduction, demonstrating the essential nature of cytochrome b5 reductase in *Arabidopsis*.

We found that all three T-DNA insertional mutants examined in *CBR1* were male gametophyte defective (Table 1). No homozygous progeny of *cbr1-3* or *cbr1-4* could be isolated, and while we were able to identify homozygous progeny of heterozygous *cbr1-2* plants, they were present at only 6% of the total progeny, substantially below the 25% frequency expected from Mendelian segregation (Table 1). When we fertilized wild-type plants with pollen from lines heterozygous for either *cbr1-3* or *cbr1-4*, a low proportion of the progeny were heterozygous for the *cbr1* allele, so some male gametophytes in these two lines are viable. There is a possibility that either *cbr1-3* or *cbr1-4* heterozygotes could segregate homozygous progeny, albeit at much lower rates than *cbr1-2*, since our limit of detection was <0.1%. No full-length *CBR1* transcript was detected in homozygous *cbr1-2* plants, even when analyzed by nested PCR amplification (see Supplemental Figure 1 online). The stable partial transcript that we detected (Figure 1; see Supplemental Figure 1 online) is unlikely to encode a functional protein, although this possibility cannot be excluded. The complementation of *cbr1-2*, *cbr1-3*, and *cbr1-4* by a *CBR1* genomic clone (see Supplemental Tables 3 and 4 online) demonstrates that the described mutant phenotypes represent loss of function of the *CBR1* gene. Isolation of homozygous *cbr1-2* plants (Figure 1B) permitted further analysis of the function of *CBR1* in *Arabidopsis*.

**CBR1 Is Substantially Redundant during Vegetative Growth**

*CBR1* is an integral membrane protein of the ER that provides reductant to desaturases and other enzymes via cytochrome b5 (Fukuchi-Mizutani et al., 1999). According to immunolocalization in BY-2 cells, *Arabidopsis* CBR1 localizes to the ER membrane (Shockey et al., 2005) and has been identified in the ER

![Figure 6. Characterization of cbr1-2 Siliques and Seed Set.](image)

(A) Siliques of the wild type (WT) and *cbr1-2*.
(B) Dissected siliques of the wild type and *cbr1-2* showing reduced seed set in the mutant.
(C) Fertilized ovules (green seeds) and unfertilized (white and shriveled) ovules were counted from dissected siliques depicted in (B). Data are mean ± se for 15 siliques.

[See online article for color version of this figure.]

**Figure 7. Outcrossing Frequency for Wild-Type and cbr1-2 Plants.**

The wild type (WT) and mutant were interplanted with transgenic plants homozygous for the DsRed fluorescent marker. The numbers of fluorescent seeds and total seeds per plant were counted at harvest. Presence of the *cbr1-2* insertion was confirmed by Basta selection of fluorescent seed derived from that line. Data are mean ± se for eight plants.
CBR1 Is Necessary for Proper Seed Maturation and Germination

The observed decrease of 18:3 and increase of 18:1 in the mature seed of cbr1-2 plants (Figure 2D) was not itself surprising, since CBR1 supplies electrons via cytochrome b5 to the FAD2 and FAD3 desaturases responsible for synthesis of 18:3 (Smith et al., 1990; Browse et al., 1993). These findings conform to analysis of the hypomorphic cbr1-1 mutant (Kumar et al., 2006). Since the fatty acid compositions of cbr1-2 leaves and roots were similar to the wild type (see Supplemental Table 2 online), we suspect that the low 18:3 in the seeds is due to the high demand for reductant imposed by fatty acid desaturation during oil accumulation in developing seeds; the lower rates of fatty acid synthesis in the leaf and root tissue permit their composition to remain unaffected. In photosynthetic tissue, the plastidial desaturases FAD7 and FAD8, which use ferredoxin as reductant, can also compensate for the reduced flux through the ER desaturases (Browse et al., 1993). In any tissue, reductant may also come via NADPH:P450 reductase isozymes; the minimal changes in root fatty acid composition in particular support the hypothesis that CBR1 is substantially redundant with P450 reductase proteins in vegetative tissues.

Although the requirement of cbr1-2 seeds for an exogenous carbon source to germinate (Figure 5A) is reminiscent of the Suc-dependent class of mutants that either fail to hydrolyze fatty acids from triacylglycerol or fail to breakdown the fatty acids via β-oxidation in the peroxisome (Hayashi et al., 1998; Fulda et al., 2004; Eastmond, 2006), it is more likely that the low germination and establishment rates were caused by a decrease in total fatty acid content in cbr1-2 seeds (Figure 2) or by a deficiency in a CBR1-related function distinct from triacylglycerol metabolism. The reduced oil, shriveled seeds, and delayed germination of cbr1-2 seeds (Figures 2 and 4) are similar to the seed phenotypes of the tag1 mutant, deficient in diacylglycerol acyltransferase activity (Routaboul et al., 1999). However, the delayed germination of tag1 seeds is not affected by Suc, and tag1 seeds showed >98% germination after 9 d of incubation on medium with or without Suc. Nevertheless, we consider it likely that the reduced oil of cbr1-2 seeds contributes, in some degree, to the germination and seedling establishment phenotypes.

CBR1 Is Required for Function of the Male Gametophyte

Segregation of the cbr1 insertional mutants at ratios of 1:1 (heterozygous:wild type) (Table 1) indicated a gametophytic
defect, and reciprocal backcrosses of heterozygous \textit{cbr1} mutant lines with the wild type pinpointed the defect in the male gametophyte. At this critical stage of the plant life cycle, compensation for loss of \textit{CBR1} activity is evidently insufficient to maintain cellular function. The gametophytic defect cannot be solely the result of reduced 18:3 fatty acid, since \textit{Arabidopsis fad3} mutant plants have greatly reduced 18:3 but produce normal gametophytes (Browse et al., 1993), so the observed defect must be due to the loss of \textit{CBR1} electron supply to enzymes other than the fatty acid desaturases. The enzymes affected by \textit{CBR1} deficiency might even be P450 monoxygenases, since cytochrome b5 has been shown to contribute electrons to P450 3',5'-hydroxylases involved in flavonoid metabolism in petunia (\textit{Petunia hybrida}; de Vetten et al., 1999) and grape (\textit{Vitis vinifera}; Bogs et al., 2006).

The male gametophyte defect revealed by segregation analysis of all three insertion mutants, coupled with the low seed set of the sole insertion line that produced homozygous plants, led us to examine pollen function in \textit{cbr1-2} plants. Alexander’s stain indicated that viability of \textit{cbr1-2} pollen grains at anthesis was comparable to the wild type (Figure 8A), and when \textit{cbr1-2} pollen was applied to either \textit{cbr1-2} or wild-type anthers, many pollen grains germinated. However, nearly all pollen tube growth ceased soon after germination and only a very few pollen tubes grew through the transmitting tract to complete fertilization (Figures 8 and 9). The process of fertilization in \textit{Arabidopsis} unfolds in stages after a pollen grain alights on a papillary cell of the stigma (Palanivelu and Tsukamoto, 2012). Like wild-type pollen, when pollen of \textit{cbr1-2} plants contacted the stigmas of wild-type or mutant plants, germination appeared robust, and pollen tubes began extension into the stigma tissue, but ceased shortly thereafter.

For pollen of \textit{cbr1-2} plants, only rarely did pollen tubes penetrate the style and proceed along the transmitting tract to effect fertilization, whether these pollen were transferred naturally (Figure 8) or by hand (Figure 9). This pattern bears some similarity to the male reproductive defect seen in plants mutant in the pectin methylesterase homolog \textit{VANGUARD1}, whose pollen tubes penetrate the style much more slowly than wild-type tubes (Jiang et al., 2005). Pollen from \textit{pip5k4} mutants, defective in phosphatidylinositol-4-monophosphate 5-kinase4, produce pollen tubes that take about twice as long as the wild type to achieve fertilization (Sousa et al., 2008). Defects like these that slow pollen tube growth often result in seed predominately at the top of the silique (Muralla et al., 2011), a pattern not discernible in \textit{cbr1-2} mutants, whose few seeds appeared randomly distributed throughout the length of the silique (Figure 6). Some mutations causing slow pollen tube growth fail to exit the transmitting tract and target ovules for fertilization. \textit{Arabidopsis} plants mutant in TFIIB1, a general transcription factor (Zhou et al., 2013), or mutant in a homolog to the animal translationally controlled tumor protein (Berkowitz et al., 2008), both show defects in targeting ovules; however, no such defect is evident in the \textit{cbr1-2} mutant, which successfully fertilized ovules when the pollen tubes traversed the transmitting tract (Figures 8 and 9).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9.png}
\caption{Reciprocal Crosses Illustrate the Gametophytic Defect in \textit{cbr1-2}.}
\textbf{(A)} Aniline blue staining of manual crosses, 24 h after pollination. WT, the wild type.
\textbf{(B)} Siliques from manual crosses, 9 d after pollination.
\textbf{(C)} Seeds per silique from manual crosses ($n = 8$); error bars indicate SE. Crosses labeled as female \times male in (A) to (C).
\end{figure}
Is a Single Cytochrome b5–Dependent Enzyme Responsible for the cbr1-2 Phenotype?

Our lipid analyses of vegetative tissue of the cbr1-2 mutant indicate that the many cytochrome b5–dependent reactions required for the synthesis of membrane glycerolipids and sphingolipids are maintained, presumably because the ATR1 and ATR2 NADPH:P450 reductase enzymes maintain adequate reduction of the cytochrome b5 pool. The low 18:3 found in the seed oil and seed phospholipids, together with the seed phenotypes we observed, indicate that cytochrome b5 reduction is partially compromised during seed development. However, it is the defect in pollen of cbr1-2 (Figures 8 and 9), cbr1-3, and cbr1-4 (Table 1) that is the key failure in the lifecycle of the mutants. We therefore considered whether electron supply from cytochrome b5 to a single, critical enzyme of membrane lipid synthesis might cause the defect in pollen tube growth observed in cbr1-2.

In glycerolipid metabolism, the major cytochrome b5–dependent reactions are those performed by the endoplasmic reticulum 18:3 desaturase, FAD3, and the 18:2 desaturase, FAD2. However, the fad3 and fad2 mutants both produce functional pollen (Miqael and Browse, 1992; Browse, et al., 1993), indicating that an effect of the cbr1-2 mutation on either of these enzyme activities cannot, by itself, explain the pollen defect. The sphingolipid Δ4desaturase (At4g04930) is highly expressed in flowers and pollen, but characterization of two knockout insertion mutants showed no difference in pollen viability, relative to the wild type (Michaelson et al., 2009). The sphingolipid Δ8 desaturase is encoded by two genes, sld1 and sld2, but double mutants with no detectable Δ8 desaturation reproduce normally and have relatively mild phenotypes (Chen et al., 2012). Similarly, mutants defective in the ACYL DESATURASE2 (ADS2) gene encoding 24:0/26:0 n-9 desaturase (Smith et al., 2013) reproduce normally and exhibit a vegetative phenotype only at low temperatures (Chen and Thelen, 2013). The FATTY ACID HYDROXYLASE1 (FAH1) and FAH2 genes encode enzymes that α-hydroxylate the fatty acids on sphingolipids (König et al., 2012; Nagano et al., 2012). fah1 fah2 double mutant plants show moderately reduced growth and other phenotypes but no indication of an effect on reproduction (König et al., 2012). A caveat to this observation is that the fah1 allele used in the cross is severe but not completely null. The sphingoid base hydroxylase1 (sbh1) sbh2 double mutants lacking sphingoid base hydroxylase do have strong phenotypes, with homozgyous double mutant seedlings showing very slow growth and a failure to progress from vegetative to reproductive growth (Chen et al., 2008). However, the double mutants were obtained in the expected Mendelian ratio from a SBH1/sbh1 SBH2/sbh2 parent, indicating that sbh1/sbh2 pollen had no significant deficiency relative to wild-type or SBH1 SBH2 (or SBH1 sbh2 or sbh1 SBH2) pollen produced by the parent plant. Other pathways of cellular biochemistry are not so completely defined, but we could find no reports of any cytochrome b5–dependent enzyme in Arabidopsis or other plant species for which a gene mutation has been shown to confer a male gametophyte lethal phenotype (Murala, et al., 2011).

While deficiencies in the individual cytochrome b5–dependent enzymes discussed here apparently do not affect pollen function, it is possible that greatly decreased activity of several or all of these enzymes provide combined effects on membrane structure and function that result in the defect in pollen tube growth we observed. Alternatively, we cannot exclude the possibility that an as yet uncharacterized, cytochrome b5–dependent enzyme is responsible. Nevertheless, the relatively normal vegetative growth of cbr1-2 plants supports the notion that sphingoid base hydroxylase as well as other cytochrome b5–dependent enzymes are provided electrons through the ability of NADPH:P450 reductase to reduce cytochrome b5 (Fukuchi-Mizutani et al., 1999). We conclude that the deficiency of cytochrome b5 reductase is masked in most tissues and organs of the cbr1-2 mutant by the activity of NADPH:P450 reductase encoded by ATR1 and ATR2. In pollen, and to a lesser extent in developing seeds, the P450 reductase isoforms apparently fail to adequately maintain reduction of the cytochrome b5 pool, resulting in a pleiotropic effect on cytochrome b5 enzymes.

METHODS

Plant Growth Conditions

Arabidopsis thaliana (ecotype Columbia-0) wild-type and mutant plants were cold-stratified for 2 d at 4°C, unless otherwise specified, and germinated on 1× Murashige and Skoog medium (Sigma-Aldrich) supplemented with 1% (w/v) Suc and 0.75% agar, under 16 h day/8 h night at 22°C, with a 80 μmol m−2 s−1 light intensity. Seedlings were 10 to 12 d old were transferred to soil (Pro-Mix; Premier Tech Horticulture) and grown under 24-h light at 22°C with 200 μmol m−2 s−1 light at rosette height. Resistance to fluoosinate (Basta; Bayer) was determined by watering plants twice prior to bolting, 3 to 4 d apart, with 0.011% Basta.

Identification of cbr1 Mutants

The insertion site for SALK_129878 is immediately upstream of the CBR1 open reading frame and lines homozygous for the insertion expressed the full-length CBR1 cDNA. The insertion site for sail_579_A02 is reportedly in the first intron of the CBR1 gene, but no insertion was detectable under standard PCR conditions. These two insertions were not further analyzed. The T-DNA insertional lines SAIL_644_A11 (Sessions et al., 2002) and WiscDsLox 377-380021 (Woody et al., 2007) were obtained from the ABRC at Ohio State University, and Gabi-Kat Line 371A08 was obtained from Professor Bernd Weisshaar (Universität Bielefeld, Germany) (Rosso et al., 2003). Plants were genotyped by PCR using separate primers from Professor Bernd Weisshaar Gabi-Kat at Universität Bielefeld, Germany (Rosso et al., 2003). Plants were genotyped by PCR using separate reactions with three primers, left border of the T-DNA insertion of cbr1 (primers LB3, WiscLB, or GKLK; see Supplemental Table 1 online) and a CBR1 gene-specific primer pair (primers P1 and P2 for cbr1-2 and cbr1-4 and P3 and P4 for cbr1-3; see Supplemental Table 1 online).

To test for transcript in cbr1-2, RT-PCR was performed by isolating RNA with Trizol reagent (Life Technologies) from leaf and inflorescence, followed by DNasel treatment with the DNA-free RNA kit (Zymo Research). cDNA was made using the Superscript III first-strand synthesis system (Life Technologies) and amplified with designated primers for 30 cycles of PCR.

When Gabi-Kat line 371A08 seeds were sown on Murashige and Skoog–agar supplemented with 1% (w/v) Suc and sulfadiazin, the seedlings were 98% resistant; the infrequent occurrence of wild-type plants indicates three unlinked insertion sites. A heterozygous plant was backcrossed into wild-type Arabidopsis (Columbia-0), and F2 CBR1/cbr1-4 plants from this backcross were self-pollinated. A line chosen for further analysis produced progeny that were 1:1 wild type: sulfadiazine resistant; these resistant plants were found to be heterozygous for the cbr1-4 allele.
Lipid Analysis

Fatty acids of mature seeds (20 to 50 seeds) were derivatized to fatty acid methyl esters in 1 mL of 2.5% (v/v) sulfuric acid in methanol for 1.5 h at 80°C (Miqel and Browse, 1992). Fatty acid methyl esters were extracted into hexane and analyzed by gas chromatography with flame ionization detection on a wax column (EC Wax; 30 m × 0.53 mm i.d. × 1.20 μm; Alltech) with parameters as follows: 220°C for 2 min followed by a ramp to 245°C at 10°C min⁻¹, with a 4-min final temperature hold. Total seed oil was quantified according to Li et al. (2006), except 20 seeds were used per sample instead of 50 seeds.

Lipids were extracted from leaf tissues according to Welli et al. (2002). Total lipids from mature seeds were extracted following the protocol from the Kansas Lipidomics Research center (http://www.k-state.edu/lipid/lipidomics/). Seed lipids were fractionated on a silica column, eluting the neutral lipids with 5 mL of chloroform/methanol (99:1, v/v) and the polar lipids with 5 mL of chloroform/methanol/water (5:5:1, v/v/v), followed by 2 mL chloroform and 2 mL 0.88% KCl. Polar lipids were washed with 2 mL of chloroform, dried under nitrogen, and resuspended in 200 μL of chloroform. Thin layer chromatography was used to separate polar lipids, with double development in acetone/toluene/water (91:30.8: v/v/v) on K6 Silica Gel 60A plates (Whatman) that were damped in 0.15 M ammonium sulfate and dried before use. Separated lipids were stained with 0.005% primuline in 80% acetone, visualized under UV light, and identified by comigration with standards. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol silica bands were collected and fatty acid methyl esters analyzed as described above, after addition of a known amount of 15:0 internal standard. Total leaf lipids were separated using the same thin layer chromatography, and the major phospholipid and galactolipid bands analyzed as described. Methods for extraction, separation, and analysis of sphingolipids were as described by Markham and Jaworski (2007).

Complementation Cloning

For trans-complementation of cbr1 mutants with a genomic clone, an ~3-kb fragment of genomic DNA containing the CBR1 promoter, CBR1 gene, and CBR1 3′ untranslated region was amplified using primers with NotI restriction sites (primers CBR1-PRM-topo and CBR1-PRM-end (see Supplemental Table 1 online)). This fragment was blunt-end cloned into pCRScript-Amp (Agilent Technologies) following the manufacturer's instructions and then digested with NotI and the genomic fragment cloned into the NotI site of plant transformation vector pART27 (Gleave, 1992). The resulting construct was transformed into Agrobacterium tumefaciens strain GV3101 and transgenic plants generated by floral dip (Clough and Bent, 1998). Pollen from T1 plants was hydrated in water or germinated in vitro on solid medium (Boavida and McCormick, 2007) for 16 h before staining in GUS assay buffer (0.1 M sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.5 mM potassium ferriyanide, 0.5 mM potassium ferrocyanide, 1 mM X-glucuronide, and 0.1% [v/v] Triton X-100) for 24 h at 37°C. The stained pollen was visualized as described above.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: CBR1, AED92466.1, AT5G17770; CBR2, P83291, AT5G20080; CBR3, AT1G26340, AT2G32720, AT2G46650, AT2G48810, AT5G35360, AT1G60660; ATR1, AT4G24520; ATR2, AT4G30210; FAD2, AT3G12120; FAD3, AT2G29890; D4-DES, AT4G04930; SLD1, AT3G61580; SLD2, AT2G6210; ADS2, AT2G31360; FAH1, AT2G34770; FAH2, AT4G20870; SBH1, AT1G69640; and SBH2, AT1G14290.

Supplemental Data

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

Supplemental Figure 1. Further RT-PCR Analysis of Homozygous cbr1-2.

Supplemental Figure 2. Fatty Acid Compositions of Major Seed Phospholipids.

Supplemental Figure 3. Fatty Acid Compositions of Major Leaf Glycerolipids.

Supplemental Figure 4. Compositional Analyses of the Long-Chain Base and Fatty Acid Components of Sphingolipids from Leaves of the Wild Type and cbr1-2.

Supplemental Table 1. List of Primers.

Supplemental Table 2. Fatty Acid Composition of Total Wild-Type and cbr1-2 Leaf and Root Tissues.

Supplemental Table 3. Complementation of Homozygous cbr1-2 Restores 18:3 Levels in Mature T2 Seeds.

Pollen Analysis

Pollen viability was assayed using Alexander’s staining (Alexander, 1969) and visualized on a Leica DM2000 microscope and images captured with a Leica DFC295 camera. Pollen was germinated in vitro by the method of Boavida and McCormick (2007). Pollen tubes growing in vivo were stained with aniline blue following sample preparation (Mori et al., 2006); the aniline blue staining solution was 0.1% aniline blue, 10 mM CHES buffer, pH 10, and 100 mM KCl. Stained pistils were observed with a Leica D CHIP upright microscope equipped with water immersion lenses not corrected for cover slips (HCX Plan-AP0U-V.1 series) and images captured with a Leica DFC300 FX cooled charge-coupled device camera.

GUS Assay

The CBR1 promoter was cloned into pENTR (Invitrogen) using primers CBR1-PRM-topo and CBR1-PRM-end (see Supplemental Table 1 online) and subcloned into plant transformation vector pMD162 (Curts and Grossniklaus, 2003) upstream of the GUS gene with Clonase I (Invitrogen). The resulting construct was transformed into Agrobacterium strain GV3101 and transgenic plants generated by floral dip (Clough and Bent, 1998). Pollen from T1 plants was hydrated in water or germinated in vitro on solid medium (Boavida and McCormick, 2007) for 16 h before staining in GUS assay buffer (0.1 M sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.5 mM potassium ferriyanide, 0.5 mM potassium ferrocyanide, 1 mM X-glucuronide, and 0.1% [v/v] Triton X-100) for 24 h at 37°C. The stained pollen was visualized as described above.
Supplemental Table 4. Complementation of the cbr1-3 and cbr1-4 Mutants.

ACKNOWLEDGMENTS

We thank Michael Knoblauch and staff of the Franceschi Microscopy and Imaging Center at Washington State University for advice and assistance with microscopy techniques. We also thank Deirdre Fahy for her revisions of the figures. This work has been funded by the USDA-National Institute for Food and Agriculture Competitive Grants Program (Grant 2010-65115-20393) and by the Agricultural Research Center at Washington State University. L.L.W. was partially supported by NIH, National Institute for General Medical Sciences through institutional training Grant T32-GM008336.

AUTHOR CONTRIBUTIONS

L.L.W., J.G.W., J.E.M., and J.B. designed the research. L.L.W., J.E.M., and R.K. performed the experiments and analyzed the data. L.L.W., J.G.W., and J.B. wrote the article.

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Supplemental Data for: Cytochrome b5 Reductase Encoded by CBR1 is Essential for a Functional Male Gametophyte in Arabidopsis
Laura L. Wayne, James G. Wallis, Rajesh Kumar, Jonathan E. Markham and John Browse
Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340.

Supplemental Figure 1. Further RT-PCR Analysis of Homozygous cbr1-2.

(A) Nested RT-PCR of cbr1-2 leaf and inflorescence (Inflores.) tissues. Secondary PCR (2°) used 20-fold dilution of primary RT-PCR (1°) products. Control amplification of ACT8 demonstrates that the RNA was intact.

(B) RT-PCR of cbr1-2 using CBR1 P6 primer (see Figure 1) and a series of primers designed to the left border of the T-DNA insertion (primers described in Supplemental Table 4, online), amplifying up to 1300bp within the T-DNA. Right lane, molecular weight markers. In the left lane, no template was added to the reaction.
In Supplementary Figures 2 - 4 the fatty acid are designated X:Y where X is the number of carbon atoms in the acyl chain and Y the number of C=C double bonds. In Supplemental Figure 4 the sphingolipid longchain bases are designated:
d18:0, dihydroxy 18-carbon chain with no desaturation (sphinganine)
t18:0, trihydroxy 18-carbon chain with no desaturation (phytosphingosine)
d18:1 and t18:1, indicate desaturation at the Δ4 or Δ8 position in the carbon chain (Markham, et al., 2013)
**Supplemental Figure 3.** Fatty Acid Compositions of Major Leaf Glycerolipids.

Individual lipids were separated from extracts of rosette leaf tissue from wild-type (white bars) and cbr1-2 (black bars). The amount of each phospholipid (µg/g fresh weight +/- SD) for wild type (WT) and cbr1-2 are listed below.

(A) Phosphatidylcholine, WT, 492+/−12; cbr1-2, 523+/−95
(B) Phosphatidylethanolamine, WT, 265+/−13; cbr1-2, 277+/−59
(C) Phosphatidylglycerol, WT, 328+/−33; cbr1-2, 333+/−46
(D) Monogalactosyldiacylglycerol, WT, 1463+/−157; cbr1-2, 706+/−48
(E) Digalactosyldiacylglycerol, WT, 679+/−13; cbr1-2, 998+/−5

(In phosphatidylglycerol 16:1 is the Δ3, trans isomer.)
Supplemental Figure 4. Compositional Analyses of the Longchain Base (LCB) and Fatty Acid Components of Sphingolipids from Leaves of Wild Type (white bars) and cbr1-2 (black bars). The amount of each sphingolipid (nmole/g dry weight +/- SD) for wild type (WT) and cbr1-2 are listed below.

(A,B) Total sphingolipids
(C,D) Glycosyl inositolphosphoceramide (GIPC), WT, 384+/−109; cbr1-2, 437+/−130
(E,F) Glucosylceramide, WT, 175+/−6; cbr1-2, 169+/−48
(G,H) Ceramide, WT, 66+/−6; cbr1-2, 115+/−10
(I,J) Hydroxyceramide, WT, 56+/−8; cbr1-2, 69+/−14

The fatty acids shown include α-hydroxy derivatives, which predominate in glucosylceramides and GIPCs.
**Supplemental Table 1.** List of Primers.

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<th>Primer</th>
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<td>CBR1-PRM-end</td>
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* Not1 restriction site nucleotides are underlined.
### Supplemental Table 2. Fatty Acid Composition of Total WT and cbr1-2 Leaf and Root Tissues.

<table>
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<th>Fatty Acids</th>
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</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>15.0±0.2</td>
<td>15.2±0.2</td>
<td>25.7±0.7</td>
<td>22.8±0.6</td>
</tr>
<tr>
<td>16:1</td>
<td>3.2±0.0</td>
<td>2.7±0.2</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>16:2</td>
<td>0.5±0.0</td>
<td>0.5±0.1</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>16:3</td>
<td>12.8±0.1</td>
<td>13.2±0.1</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>18:0</td>
<td>1.5±0.0</td>
<td>1.4±0.0</td>
<td>5.3±0.8</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>18:1</td>
<td>1.7±0.0</td>
<td>1.5±0.2</td>
<td>5.6±0.3</td>
<td>11.4±1.2</td>
</tr>
<tr>
<td>18:2</td>
<td>12.8±0.2</td>
<td>12.4±1.0</td>
<td>42.1±1.0</td>
<td>42.3±0.6</td>
</tr>
<tr>
<td>18:3</td>
<td>52.5±0.4</td>
<td>53.1±1.4</td>
<td>21.4±0.2</td>
<td>20.2±0.3</td>
</tr>
</tbody>
</table>

Fatty acids were derivatized directly from leaf and root tissues. Data are means +/- SE for three biological replicates. n/d, not detected, <0.5%.

### Supplemental Table 3. Complementation of Homozygous cbr1-2 Restores 18:3 Levels in Mature T_2_ Seeds.

<table>
<thead>
<tr>
<th>Line</th>
<th>Fatty Acid (relative %)</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:0</th>
<th>20:1</th>
<th>20:2</th>
<th>22:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>cbr1-2 +CBR1 #7</td>
<td>8.4</td>
<td>0.4</td>
<td>2.7</td>
<td>18.0</td>
<td>30.9</td>
<td>17.7</td>
<td>1.8</td>
<td>17.3</td>
<td>1.5</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>cbr1-2 +CBR1 #12</td>
<td>8.4</td>
<td>0.4</td>
<td>2.8</td>
<td>14.6</td>
<td>29.6</td>
<td>21.2</td>
<td>2.0</td>
<td>17.9</td>
<td>1.8</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>cbr1-2</td>
<td>9.0</td>
<td>0.6</td>
<td>3.8</td>
<td>28.8</td>
<td>30.5</td>
<td>5.7</td>
<td>2.1</td>
<td>17.7</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>cbr1-2</td>
<td>10.1</td>
<td>0.7</td>
<td>3.5</td>
<td>29.3</td>
<td>30.8</td>
<td>7.3</td>
<td>2.0</td>
<td>15.7</td>
<td>0.7</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>8.2</td>
<td>0.4</td>
<td>2.7</td>
<td>14.7</td>
<td>30.3</td>
<td>20.7</td>
<td>1.9</td>
<td>17.8</td>
<td>1.8</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>8.6</td>
<td>0.4</td>
<td>2.8</td>
<td>16.0</td>
<td>30.1</td>
<td>20.2</td>
<td>1.9</td>
<td>17.3</td>
<td>1.6</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

Homzygous cbr1-2 was transformed with the CBR1 genomic clone (+CBR1) and compared with the parental cbr1-2 and WT. Two representative lines (from 16 total) are shown.
**Supplemental Table 4.** Complementation of The cbr1-3 and cbr1-4 Mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Progeny</th>
<th>Line</th>
<th>cbr1/cbr1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBR1/cbr1-3</td>
<td></td>
<td>3-3</td>
<td>5</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+CBR1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-CBR1</td>
<td>0</td>
</tr>
<tr>
<td>CBR1/cbr1-3</td>
<td></td>
<td>3-4</td>
<td>6</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+CBR1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-CBR1</td>
<td>0</td>
</tr>
<tr>
<td>CBR1/cbr1-4</td>
<td></td>
<td>45-1</td>
<td>8</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+CBR1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-CBR1</td>
<td>0</td>
</tr>
</tbody>
</table>

Heterozygous plants were transformed with the CBR1 genomic clone and the progeny genotyped by PCR. <sup>a</sup>+CBR1 indicates progeny containing the genomic transgene.