The γ-Carbonic Anhydrase Subcomplex of Mitochondrial Complex I Is Essential for Development and Important for Photomorphogenesis of Arabidopsis1[C][W][OA]

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Complex I (NADH:ubiquinone oxidoreductase) is the entry point for electrons into the respiratory electron transport chain; therefore, it plays a central role in cellular energy metabolism. Complex I from different organisms has a similar basic structure. However, an extra structural module, referred to as the γ-carbonic anhydrase (γCA) subcomplex, is found in the mitochondrial complex I of photoautotrophic eukaryotes, such as green algae and plants, but not in that of the heterotrophic eukaryotes, such as fungi and mammals. It has been proposed that the γCA subcomplex is required for the light-dependent life style of photoautotrophic eukaryotes, but this hypothesis has not been successfully tested. We report here a genetic study of the genes γCAL1 and γCAL2 that encode two subunits of the γCA subcomplex of mitochondrial complex I. We found that mutations of γCAL1 and γCAL2 in Arabidopsis (Arabidopsis thaliana) result in defective embryogenesis and nongerminating seeds, demonstrating the functional significance of the γCA subcomplex of mitochondrial complex I in plant development. Surprisingly, we also found that reduced expression of γCAL1 and γCAL2 genes altered photomorphogenic development. The γcal1 mutant plant expressing the RNA interference construct of the γCAL2 gene showed a partial constitutive photomorphogenic phenotype in young seedlings and a reduced photoperiodic sensitivity in adult plants. The involvement of the γCA subcomplex of mitochondrial complex I in plant photomorphogenesis and the possible evolutionary significance of this plant-specific mitochondrial protein complex are discussed.

The light-dependent development of plants, or photomorphogenesis, has been extensively studied in Arabidopsis (Arabidopsis thaliana). It is now clear that several nuclear photosensory receptors, such as red/far-red light receptor phytochromes, blue light receptor cryptochromes, and LOV domain F-box proteins ZEITLUPE (ZTL) and FLAVIN-BINDING, KELCH (FBK) interact with transcription regulators of transcription or protein degradation to affect gene expression. For example, phytochromes interact with the basic helix-loop-helix (bHLH) transcription factor PHYTOCHROME-INTERACTING FACTORS (PIFs) in response to red/far-red light to trigger degradation of the PIF proteins and alter the transcription of the PIF target genes (Ni et al., 1998; Huq and Quail, 2002; Bauer et al., 2004; Leivar and Quail, 2011); cryptochromes interact with the bHLH transcription factor CRYPTOCHROME-INTERACTING BHLH1 and the SUPPRESSOR OF PHYTOCHROME A1/COP1 E3 ubiquitin ligase complex in response to blue light to regulate transcription and protein degradation, respectively (Yang et al., 2000; Wang et al., 2001; Liu et al., 2008, 2011a; Lian et al., 2011; Zuo et al., 2011); and ZTL and FKFI interact with transcription regulators such as TIMING OF CAB EXPRESSION1, CYCLING DOF FACTOR1, GIGANTEA, and CONSTANS (CO) to modulate the transcription or protein degradation of the target genes and physiological responses to light (Más et al., 2003; Imaizumi et al., 2005; Kim et al., 2007; Sawada et al., 2007; Ito et al., 2012). Many photoreceptor target...
genes are regulators or enzymes of various metabolic processes, especially the energy metabolism in chloroplasts and mitochondria (Ma et al., 2001). For example, Arabidopsis cryptochromes regulate the transcription of the nucleus-encoded chloroplast σ factor, SIG5, to regulate plastid transcription and development to affect photomorphogenesis (Ruckle et al., 2007; Ruckle and Larkin, 2009). However, it is less clear how photoreceptors affect mitochondrial development or whether mitochondrial proteins affect photomorphogenesis.

The respiratory complex I (NADH:ubiquinone oxidoreductase or NADH dehydrogenase) is the entry point of electrons to the mitochondrial respiratory electron transport chain. Despite the similar function in energy metabolism, complex I of photoautotrophic organisms exhibits different subunits and structure features in comparison with its counterpart of heterotrophic organisms (Brauna and Zabaleta, 2007; Hunte et al., 2010; Klodmann et al., 2010). Notably, complex I of plants and algae contains a γ-carbonic anhydrase (γCA) subcomplex that is absent from complex I of fungi and animals (Dudkina et al., 2005; Sunderhaus et al., 2006; Brauna and Zabaleta, 2007; Bultema et al., 2009; Efremov et al., 2010; Hunte et al., 2010; Klodmann et al., 2010; Klodmann and Braun, 2011; Fig. 1A). In Arabidopsis, the γCA subcomplex contains three carbonic anhydrase subunits, γCA1 (At1g19580), γCA2 (At1g47260), and γCA3 (At5g66510), and two γCA-like subunits, γCAL1 (AT5G63510) and γCAL2 (AT3G48680). The γCA and γCAL genes are widely found in plant genomes but not in animal genomes examined thus far (Brauna and Zabaleta, 2007). Therefore, it was proposed that the γCA subcomplex may play a role in the light-dependent life style of plants (Brauna and Zabaleta, 2007). Several Arabidopsis mutants have been reported that affect proteins of complex I; these mutants showed growth retardation, abnormal leaf morphology, and poor germination (de Longevialle et al., 2007; Keren et al., 2012). A tobacco (Nicotiana tabacum) complex I-specific mutant, CMSII, exhibits decreased photosynthesis at atmospheric

Figure 1. γCAL1 and γCAL2 are mitochondrial proteins of the plant-specific γCA subcomplex of complex I. A. Diagram depicting the γCA subcomplex (black module indicated by arrows), the mitochondrial complex I of green algae (Polytomella spp.) and plants (Arabidopsis; Dudkina et al., 2005; Sunderhaus et al., 2006), but not in bacteria (Escherichia coli; Morgan and Sazanov, 2008), fungi (Neurospora spp.; Guénebaut et al., 1997), or mammals (bovine; Grigorieff, 1998). The structural outlines of complex I are redrawn from the published structure of the respective complex I. B to E, Confocal images showing the subcellular localization of the γCAL1-YFP and γCAL2-YFP (B–D) or γCAL1-GFP and γCAL2-GFP (E) fusion proteins in hypocotyl cells of 5-d-old seedlings (B and C) or protoplasts isolated from 3-week-old plants (E). CS16264 is a mitochondrial marker protein. Propidium iodide (PI) was used to stain the cell wall. BF, Bright field. Bars = 20 μm (B and C) and 25 μm (D). The boxed areas (B–D) are enlarged to show details. [See online article for color version of this figure.]
CO₂ levels but not at higher CO₂ levels (Sabar et al., 2000; Dutilleul et al., 2003), demonstrating a role of complex I in light-dependent energy metabolism. However, because those complex I mutants affect either multiple complex I proteins or proteins not specific to photosynthetic organisms, how the plant-specific subunits of complex I, especially that of the CA subcomplex, might affect plant development remains unknown. Indeed, no photomorphogenic phenotype has been reported for those tobacco or Arabidopsis mutants (Sabar et al., 2000; Dutilleul et al., 2003; de Longevialle et al., 2007; Keren et al., 2012). The T-DNA insertion mutations of Arabidopsis genes encoding the CA subunits have been reported previously, but none of those mutants showed visible phenotypic alterations, making it difficult to directly test the physiological function of the CA subcomplex (Perales et al., 2005). For example, mutants impaired in the CA2 or CA3 genes exhibited morphologic phenotypes indistinguishable from that of the wild-type plants (Perales et al., 2005). A suspension culture derived from the ca2 mutant showed clearly reduced growth rate and respiration, but it remains unclear whether such a defect is dependent on light and how the phenotype of the suspension culture is directly related to specific aspects of the development of whole plants.

RESULTS

γCAL1 and γCAL2 Are Mitochondrial Proteins

We isolated clones corresponding to the Arabidopsis γCAL2 gene from a yeast two-hybrid screen in our search for proteins that interact with the blue light receptor CRY2 (Liu et al., 2008; Zuo et al., 2011; X. Yu, unpublished data). The Arabidopsis genome encodes three closely related CAs, γCAL1, γCAL2, and γCAL3, and two γCA-like proteins, γCAL1 and γCAL2, which together form the CA subcomplex of the mitochondrial complex I (Perales et al., 2004; Klodmann et al., 2010; Klodmann and Braun, 2011; Supplemental Fig. S1). The amino acid sequences of the three CA subunits contain all critical and evolutionarily conserved residues in the CAs, whereas the two γCAL subunits lack two critical His residues that are conserved in the carbonic anhydrases and, therefore, are not likely to be catalytically active (Perales et al., 2004; Klodmann et al., 2010; Supplemental Fig. S1). γCAL1, γCAL2, and γCAL3 are more closely related to each other (approximately 75% identity) than they are to γCAL1 and γCAL2 (approximately 30% identical; Supplemental Fig. S1). The γCAL1 and γCAL2 genes, which share approximately 90% amino acid sequence identity, appear to express constitutively without obvious responses to light or circadian rhythm (Supplemental Fig. S2). The γCAL1 and γCAL2 proteins have been previously identified in the Arabidopsis mitochondrial fraction by systematic mass spectrometry analyses (Sunderhaus et al., 2006; Klodmann et al., 2010). To verify the subcellular localization, we prepared and examined transgenic plants expressing γCAL1-YELLOW FLUORESCENT PROTEIN (YFP) and γCAL2-YFP proteins. We found that the γCAL1-YFP and γCAL2-YFP fusion proteins accumulate exclusively in mitochondria in the transgenic plants (Fig. 1, B–E). Given that Arabidopsis CRY2 performs all the known functions and undergoes light-dependent protein modifications in the nucleus (Yu et al., 2007), γCAL2 seems unlikely to be directly involved in the function or regulation of CRY2, and our initial yeast two-hybrid result is most likely an often-encountered artifact in such experiments. However, we cannot completely exclude the remote possibility that CRY2 and γCAL2 may interact in the cytosol before they are imported into their respective organelles. Because of a potential involvement of these mitochondrial proteins in light-dependent plant growth and development (Brauna and Zabaleta, 2007), we continued our study to investigate the physiological functions of γCAL1 and γCAL2.

γCAL1 and γCAL2 Are Essential Genes with Overlapping Functions

We first collected and analyzed a complete set of T-DNA insertion mutants of the genes encoding each of the five subunits of the Arabidopsis γCA subcomplex, including γca1, γca2, γca3, γcal1, and γcal2 (Supplemental Figs. S3 and S4). None of the monogenic mutants or the caul3 double mutant showed easily discernible phenotypic alternation (Supplemental Fig. S4). These results are consistent with previous reports on phenotypes of caul and ca3 mutants (Perales et al., 2004). Transgenic plants overexpressing the γCAL1 and γCAL2 genes also showed no apparent phenotypic alterations (X. Yu, unpublished data).

We reasoned that the γCAL1 and γCAL2 genes may have redundant functions, given that they share over 90% sequence identity (Supplemental Fig. S1), and prepared the double mutant by a conventional genetic method. However, we failed to identify the caul caul double mutant after genotyping over 500 F2 progeny derived from reciprocal crosses of caul1 and caul2 mutants (X. Yu, unpublished data). Because plants homozygous for one γCAL gene but heterozygous for the other gene (ca1ca2ca2 or ca1ca2ca2) showed a normal phenotype, we suspect that the caul caul double mutant may suffer from embryonic defects. To test this possibility, we examined young siliques derived from parents that are homozygous for the mutation of one γCAL gene but heterozygous for the other gene (ca1ca2ca2 or cuca1ca2c1). We found that young siliques derived from the caul caul or ca1ca2c1 parents contain approximately 2% to 25% colorless ovules (Fig. 2A, left), which appeared to turn deep brown at the later stage of siliques development (Fig. 2A, right). Those colorless/deep-brown ovules were apparently delayed in embryogenesis to various extents, in comparison with that of the normal green ovules (Fig. 2, B and C). A statistical analysis demonstrated that the colorless/deep-brown phenotype of the caul caul parents (23.91%) exhibits the 3:1
Segregation ratio (Table I), indicating that those abnormal ovules are the double mutant progeny of the c1c1C2c2 parent. The colorless/deep-brown phenotype of the C1c1c2c2 parents (19.94%) appears to deviate from the 3:1 segregation (Table I). These results suggest that the cal2 mutation has a nearly normal penetrance for the colorless/deep-brown ovule phenotype, whereas the cal1 mutation may have a less than normal penetrance.

Surprisingly, in contrast to a common embryonic defective mutant, we did not observe an abnormal number of aborted embryos in the mature siliques of the c1c1C2c2 and C1c1c2c2 plants (Fig. 2A, right). We reasoned that the colorless/deep-brown ovules might complete their development to set seeds, but the double mutant seeds may fail to germinate. This explains why we were unable to find the double mutant in the F2 plants. To test this interpretation, we

**Figure 2.** The γCAL1 and γCAL2 subunits of the γCA subcomplex is essential for Arabidopsis development. A, The immature siliques of wild-type (WT) and rescued mutant (35S::CAL1/c1c1c2c2) parents that show uniform and green-colored ovules, and the siliques derived from C1c1c2c2 or c1c1C2c2 hemizygote parents that show segregating colorless ovules indicated by arrowheads (left). The mature siliques from the wild-type and 35S::CAL1/c1c1c2c2 parents show uniformly yellow seeds, but those from the c1c1C2c2 hemizygote parent show segregating deep-brown seeds indicated by arrowheads (right). No dark-brown seed was found in the mature siliques of the C1c1c2c2 hemizygote parents, but no double homozygous progeny were identified. Bars = 0.5 mm. B, Embryos of the segregating progeny of the c1c1C2c2 hemizygote. Ovules from a young silique of the c1c1C2c2 hemizygote parent were cleared with Hoyer’s solution and observed with a differential interference contrast microscope. The segregating white ovules arrested at various stages (c1c1c2c2) and the pale-green wild-type-looking ovule at the mature cotyledon stage from the same young silique are shown. Bars = 50 μm. C, Seeds (left) and embryos (right) of the nongerminating seeds (ngs) harvested from the C1c1c2c2 and c1c1C2c2 hemizygote parents. For the wild type, C1c1c2c2 and c1c1C2c2 seeds were placed on MS medium for 7 d. The nongerminating seeds were collected first for photographic record (left) and then dissected using a microscope. The dissected embryos corresponding to the seed (left) are shown on the right. D, Genotyping of the nongerminating seeds. Genomic DNA was isolated from the cal1 mutant, the cal2 mutant, and nongerminating seeds derived from the c1c1C2c2 parent. Genomic PCR was performed using the primers designed to amplify the wild-type γCAL1 (CAL1) and γCAL2 (CAL2) genes or the γcal1 (cal1) and γcal2 (cal2) mutant genes. [See online article for color version of this figure.]
examined the germination rate of the seeds harvested from the selfed c1c1C2c2 and C1c1c2c2 parents (Table II). In contrast to the wild-type seeds that showed the germination rate of higher than 99%, about 20% to 25% of seeds derived from the selfed c1c1C2c2 and C1c1c2c2 parents failed to germinate (Table II). As a control, the seeds resulting from the reciprocal crosses between the wild-type and the c1c1C2c2 and C1c1c2c2 plants showed normal germination rates (Supplemental Table S1), suggesting that the nongerminating seeds derived from selfed c1c1C2c2 and C1c1c2c2 parents are double mutants. Similar to the colorless/deep-brown ovule phenotype, the cal2 mutation had a nearly normal penetrance for the nongermination phenotype, whereas the cal1 mutation had a less than normal penetrance for the germination phenotype (Supplemental Table S1).

We confirmed that the colorless ovule and nongerminating seed phenotypes both result from the parental c1c1c2c2 double mutation by genotyping and complementation tests (Figs. 2D and 3; Tables I and II). We first collected the nongerminating seeds from the progeny derived from selfed c1c1C2c2 parents and genotyped those seeds. Indeed, the nongerminating seeds derived from the c1c1C2c2 hemizygote parents contained T-DNA inserts in both the ycal1 and ycal2 mutant loci, and they lacked the intact yCAL1 and yCAL2 genes (Fig. 2D). For the complementation assay, we transformed the C1c1c2c2 plants with the 35S::CAL1 transgene and genotyped the transgenic individuals (Basta resistant) in the T2 generation (Fig. 3). All T2 Basta-resistant individuals grew normally and showed normal ovules and seed germination (Tables I and II). We randomly selected some T2 Basta-resistant individuals to identify those that showed homozygous ycal1 and ycal2 mutations (c1c1c2c2; Fig. 3A). In contrast to our previous failure in identifying any c1c1c2c2 individuals homozygous for both the ycal1 and ycal2 mutations in over several hundred progeny of the C1c1c2c2 parent, we readily identified individuals homozygous for both mutations (c1c1c2c2) in the Basta-resistant transgenic progeny of the C1c1c2c2 parent (Fig. 3A). We then examined the existence of the 35S::CAL1 transgene in plants of the c1c1c2c2 genotype and found that all c1c1c2c2 double mutant plants also contained the 35S::CAL1 transgene (Fig. 3B). This result confirmed that the c1c1c2c2 double mutant was rescued by the 35S::CAL1 transgene. We conclude that the cal1cal2 double mutation caused the defective embryogenesis and nongerminating seed phenotypes and that the yCAL1 and yCAL2 genes are essential for plant development.

### Table I. Approximately 20% to 25% of ovules of the c1c2C2c2 and C1c1c2c2 hemizygote parents show defective embryogenesis

The young silique of the indicated genotypes were dissected using a microscope to examine the ovules. The numbers of normal (green) and defective (colorless) ovules were calculated and are shown. The frequency of the colorless ovules of c1c1C2c2 (23.91%) is consistent with the 3:1 segregation ratio. The frequency of colorless ovules observed in young silique of the C1c1c2c2 hemizygote parent (19.94%) deviates from the 3:1 segregation, indicating a less than perfect penetrance of the ycal mutation. The frequency of colorless ovules observed in young silique derived from the 35S::CAL1/c1c1c2c2 transgenic plant (1.53%) is similar to that of the wild-type control (0.35%), indicating rescue of the colorless ovules of the c1c1c2c2 double mutant by the 35S::CAL1 transgene.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>cal1</th>
<th>cal2</th>
<th>c1c1c2c2</th>
<th>C1c1c2c2</th>
<th>35S::CAL1/c1c1c2c2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ovules</td>
<td>862</td>
<td>834</td>
<td>853</td>
<td>1,150</td>
<td>1,329</td>
<td>720</td>
</tr>
<tr>
<td>Green ovules</td>
<td>859</td>
<td>831</td>
<td>847</td>
<td>875</td>
<td>1,064</td>
<td>709</td>
</tr>
<tr>
<td>Colorless ovules</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>275</td>
<td>265</td>
<td>11</td>
</tr>
<tr>
<td>Colorless (%)</td>
<td>0.35</td>
<td>0.36</td>
<td>0.70</td>
<td>23.91</td>
<td>19.64</td>
<td>1.53</td>
</tr>
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\[
\chi^2 (3:1) = \frac{(23.91 - 19.94)^2}{19.94} = 0.72
\]


Decreased Expression of yCAL1 and yCAL2 Altered Plant Light Responses

Since the monogenic ycal1-1 and ycal2-1 mutants showed no phenotype alteration whereas the c1c1c2c2 double mutant failed to germinate, we sought to generate intermediate phenotypes to facilitate functional analysis using a “knockdown” approach. We prepared yCAL2 RNA interference (RNAi) transgenic lines in the wild type or the cal1 mutant backgrounds, which are referred to as c2i or c1c2i knockout lines, respectively. Both knockdown lines showed similar phenotypes (Figs. 4 and 5; Supplemental Fig. S5; data not shown), but we focused on the c1c2i knockdown lines for the following analyses (Figs. 4–7). The c1c2i knockdown lines showed reduced expression of the yCAL1 and yCAL2 genes without affecting the mRNA expression of the three yCA genes of the yCA subcomplex (Fig. 5A; Supplemental Fig. S6). The phenotypic alterations of the c1c2i knockdown plants are observed in multiple independent transgenic lines (Figs. 4–7), arguing that the phenotypic alterations described are due to the reduced expression of the yCAL1 and yCAL2 genes. Consistent with the observation that the c1c1c2c2 double mutant failed to germinate, the c1c2i knockdown lines showed delayed germination (Fig. 4). After germination, the c1c2i plants appeared to grow normally, except that they exhibited smaller stature (Fig. 5; Supplemental Fig. S5). By the time the wild-type plants developed four true leaves, the c1c2i plants had only two true leaves, and the leaves of the c1c2i plants remained markedly smaller than...
Table II. Approximately 20% to 25% of seeds of the c1c2C2c2 and C1c1c2c2 hemizygote parents failed to germinate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>call1</th>
<th>cal2</th>
<th>c1c1C2c2</th>
<th>C1c1c2c2</th>
<th>35S:CAL1/c1c2c2</th>
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</thead>
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<tr>
<td>Germinating seeds</td>
<td>514</td>
<td>746</td>
<td>593</td>
<td>454</td>
<td>467</td>
<td>627</td>
</tr>
<tr>
<td>Nongerminating seeds</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>148</td>
<td>119</td>
<td>2</td>
</tr>
<tr>
<td>Nongerminating seeds frequency (%)</td>
<td>0.39</td>
<td>0.27</td>
<td>0.17</td>
<td>24.58</td>
<td>20.31</td>
<td>0.32</td>
</tr>
</tbody>
</table>

\( \chi^2 (3:1) = 2.47 \)

In addition to the altered photomorphogenic development of young seedlings, decreased expression of \( \gamma \text{CAL1} \) and \( \gamma \text{CAL2} \) also affects photoperiod-dependent reproductive development of the knockdown plants (Fig. 7). When grown in long-day photoperiods, the \( \text{c1c2i} \) plants clearly exhibited a late-flowering phenotype measured by both “days to flower” and “rosette leaf number” (Fig. 7A). Although the \( \text{c1c2i} \) plants grown in short-day photoperiods also took longer to flower than the wild-type control as measured by days to flower, the \( \text{c1c2i} \) and wild-type plants developed the same number of rosette leaves at the time of flowering in short days (Fig. 7B). This result argues that the apparent late-flowering phenotype of the \( \text{c1c2i} \) plants grown in the short-day photoperiod is due to a retardation of growth rather than a delay in floral initiation per se (Koornneef et al., 1991). Consistent with the notion that \( \gamma \text{CAL1} \) and \( \gamma \text{CAL2} \) expression affects photoperiodic flowering, genes closely associated with the photoperiodic control of floral initiation, such as \( \text{CO} \), \( \text{SOC1} \), and \( \text{FT} \), showed markedly reduced mRNA expression in the \( \text{c1c2i} \) plants, whereas another flowering-time gene, \( \text{FLC} \), which is an autonomous pathway gene not directly involved in the photoperiodic control of flowering time, showed normal expression in the \( \text{c1c2i} \) plants (Searle and Coupland, 2004; Sung and Amasino, 2005; Baurle and Dean, 2006; Fig. 7C). Importantly, the development-dependent change of mRNA expression...
for the photoperiodic pathway genes $FT$ and $SOC1$, but not the autonomous pathway gene $FLC$, is almost abolished in the $c1c2i$ plants (Fig. 7C). Given the essential role of $\gamma$CAL1 and $\gamma$CAL2 in plant development (Figs. 1–5), this result argues that the developmental impacts of these two mitochondrial proteins affect the photoperiodic pathway more than the autonomous pathway that controls floral initiation. Taken together, we conclude that $\gamma$CAL1 and $\gamma$CAL2 play important roles in light-dependent plant growth and development.

**DISCUSSION**

We have demonstrated in this report the physiological role of a plant-specific $\gamma$CA subcomplex of mitochondrial complex I in Arabidopsis. We found that $\gamma$CAL1 and $\gamma$CAL2 are not only essential for plant development, they are also involved in the light regulation of seedling development and photoperiodic control of flowering. The association of $\gamma$CAL1 and $\gamma$CAL2 with light-dependent plant development appears to provide a functional and evolutionary explanation for why the $\gamma$CA subcomplex is found only in the mitochondrial complex I of photoautotrophic organisms. On the other hand, our study also raised several questions that remain to be further investigated.

It remains unclear how mitochondrial proteins $\gamma$CAL1 and $\gamma$CAL2 affect the light regulation of plant growth. The mRNA abundances of the nuclear genes $\gamma$CAL1 and $\gamma$CAL2 are not apparently regulated by light (Supplemental Fig. S2). It is possible that light may affect the protein modification or abundance of $\gamma$CAL1 and $\gamma$CAL2. Our attempts to raise antibodies against the $\gamma$CAL1 and $\gamma$CAL2 proteins were unsuccessful, so these hypotheses cannot be directly tested at present. Alternatively, the $\gamma$CA subcomplex may affect the composition and activity of complex I, which in turn affects energy metabolism in response to light, such as photosynthesis. Indeed, the $c1c2i$ plants exhibited a modest but statistically significant ($P < 0.05$) reduction in the photochemical efficiency of PSII, especially at a relatively high fluence rate of light (Supplemental Fig. S8). This result is consistent with the previous finding that the mitochondrial complex I is important for photosynthesis (Sabar et al., 2000; Dutilleul et al., 2003). The trivial explanation of a change in the composition of light harvesting and photosystem stoichiometry is unlikely, because the chlorophyll $a/b$ ratios are unchanged and blue native gel electrophoresis of thylakoid membranes does not reveal any differences in the formation of supercomplexes of photosystems and light-harvesting complexes (Supplemental Fig. S9). There remains the possibility that the biogenesis or maintenance (repair

**Figure 4.** The delayed germination phenotype of the $c1c2i$ knockdown seeds. Seeds of the indicated genotypes (wild type [WT], $cal1$, and $c1c2i$) were placed on MS medium, and the photographs were taken at the times indicated. Seeds or seedlings of independent $c1c2i$ mutant transgenic lines show similar phenotypes of delayed germination or retarded growth, respectively. Bars = 1 mm. [See online article for color version of this figure.]

**Figure 5.** The growth retardation phenotypes of transgenic lines expressing the $CAL2$-RNAi construct in the $cal1$ mutant background ($c1c2i$). A, Quantitative PCR showing decreased mRNA expression of the $CAL1$ (left) and $CAL2$ (right) genes in independent transgenic lines expressing the $CAL2$-RNAi construct in the $cal1$ mutant background ($c1c2i$). B, Phenotypes of the $c1c2i$ mutant transgenic seedlings grown for 7 d in a long-day (16 h of light/8 h of dark) photoperiod (left) or a short-day (8 h of light/16 h of dark) photoperiod (right). C, Phenotypes of the $c1c2i$ mutant transgenic seedlings grown for 11 d in a long-day photoperiod. WT, Wild type. [See online article for color version of this figure.]

cycle) of PSII is impacted, which is consistent with the exacerbated phenotype with a higher light fluence (Supplemental Fig. S8). The reduced photochemical efficiency of PSII of the c1c2i plants may result from an effect on photorespiration that occurs partly in the mitochondrion. It is known that complex I affects photorespiration (Sabar et al., 2000; Dutilleul et al., 2003), and it has also been proposed that the γCA subcomplex may play a role in photorespiration (Brauna and Zabaleta, 2007). The γCA subunits (γCA1–3) of the γCA subcomplex may, by converting CO₂ to HCO₃⁻, facilitate the recycling of CO₂ released at the decarboxylation step of photorespiration to the chloroplast. Therefore, we investigated whether the γCA subcomplex may affect photorespiration by testing whether the c1c2i phenotype might be rescued by a high concentration of CO₂. It is well known that a high CO₂ concentration can suppress photorespiration and photorespiration-defective phenotypes of photorespiration-defective mutants (Somerville and Ogren, 1980; Foyer et al., 2009; Peterhansel et al., 2010). However, increased CO₂ concentration failed to rescue the reduced PSII efficiency of the c1c2i plants (Supplemental Fig. S9). Consistent with this result, the c1c2i plants grown at a high level of CO₂ (5%) also showed a similar morphological phenotype as that grown in normal air (approximately 0.03% CO₂; Supplemental Fig. S10). Therefore, the reduced PSII efficiency and growth defects of the c1c2i plants may not be simply explained by the defective photorespiration. It should be noted that our result does not necessarily imply a lack of function of the γCA subcomplex in photorespiration, because all three γCA

Figure 6. The wavelength-independent hypersensitive light responses of the c1c2i knockdown seedlings. A, Representative seedling phenotypes of the indicated genotypes. Independent lines of the c1c2i seedlings and the controls were grown in the dark or in continuous blue light (25 μmol m⁻² s⁻¹), far-red light (2.5 μmol m⁻² s⁻¹), or red light (15 μmol m⁻² s⁻¹) for 5 d. Seedlings of four independent c1c2i lines (lines 1, 5, 9, and 20) are shown. B, Hypocotyl lengths of seedlings of the indicated genotypes. Seedlings were grown for 5 d under the different fluence rates indicated, and SD values are shown (n ≥ 20). C, Quantitative PCR results showing the increased expression of the light-induced CHS gene in c1c2i seedlings. Wild-type (WT) and c1c2i seedlings were grown in the dark (D7–D9) or under continuous blue light (B7–B9) for 7, 8, or 9 d before sample harvest for RNA isolation. The relative expression of CHS is normalized by UBQ5. [See online article for color version of this figure.]
genes are apparently expressed normally in the c1c2i mutant (Supplemental Fig. S5).

The biochemical function that γCAL may play in complex I is not clear at present, nor is it clear why the reduced level of γCAL1 and γCAL2 proteins affect photomorphogenesis. As expected, γCAL2 showed no carbonic anhydrase activity (Supplemental Fig. S11A). On the other hand, it is known that the γCAs contain the hexapeptide repeats ([LIV]-X2-[STAV]-X) that form a left-handed β-helix structure (Gaedeke et al., 2001; Perales et al., 2004; Ferry, 2010). Sequence analysis and structural modeling indicate that both γCAL proteins contain the hexapeptide repeats and the left-handed β-helix folds (Supplemental Fig. S11B). The left-handed β-helix structure is widely found in members of the acyltransferase superfamily (Raetz and Roderick, 1995). This superfamily contains enzymatically unrelated proteins, including acetyltransferases, such as Ser acetyltransferases of bacteria and mitochondrial Ser acetyltransferases of Arabidopsis (Parisi et al., 2004; Ferry, 2010).
Pye et al., 2004; Haas et al., 2008). The left-handed β-helix structure constitutes the active site of the acetyltransferases (Pye et al., 2004). Interestingly, γCAL2 appears to exhibit a protein acetyltransferase activity in vitro (Supplemental Fig. S11C). The in vivo substrate of γCAL2 remains unclear at present. Our attempts to test a possible acetyltransferase activity with other subunits of complex I was unsuccessful, which may be due to the difficulties in the purification of native subunits of this membrane complex. Given that fatty acids of seed lipids are converted to acetyl-CoA during germination for the synthesis of compounds essential for germination and early seedling growth, a potential acetyltransferase activity of γCAL2, which utilizes acetyl-CoA as a substrate in vitro, may partially explain the germination defect of the yad1γcal2 double mutant. It is conceivable that the energy metabolism is important for photomorphogenesis in general, and certain malfunctions in mitochondria may adversely affect photomorphogenesis. Specifically, it is tempting to speculate that γCAL proteins may acetylate other mitochondrial proteins in response to the changing light environment and acetyl-CoA concentration to indirectly affect plant photomorphogenesis. This proposition, however, remains to be further investigated.

MATERIALS AND METHODS
Most Arabidopsis (Arabidopsis thaliana) yca and ycal mutants and all transgenic lines described in this study are from the Columbia accessions, except the γcal2 mutant, which is from the Wassilewskija accession. All the mutants were obtained from the Arabidopsis Biological Resource Center. Plant transformation and genetic, photomorphogenic, DNA, and RNA analyses were as described previously (Yu et al., 2007; Liu et al., 2008; Li et al., 2011). For the embryonic phenotype analyses, young (green) and mature (brown) siliques (15–20 per sample) were collected from 3- to 4-week-old plants. For the embryonic phenotype analyses, young (green) and mature (brown) siliques (15–20 per sample) were collected from 3- to 4-week-old plants. For the embryonic phenotype analyses, young (green) and mature (brown) siliques (15–20 per sample) were collected from 3- to 4-week-old plants.

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

Supplemental Figure S1. Sequence analysis of the γCAL and γCA proteins.

Supplemental Figure S2. Light-independent mRNA expression of γCAL1 and γCAL2.

Supplemental Figure S3. Genotyping of the ycal1 and ycal2 mutants.

Supplemental Figure S4. Phenotypes of adult plants of the indicated genotypes.

Supplemental Figure S5. mRNA expression of the γCA genes are not affected in the c1c2i knockdown plants.

Supplemental Figure S6. Analyses of transgenic lines expressing the CAL2-RNAi construct in the wild-type background (c2i).

Supplemental Figure S7. Morphological defects of leaves of the c1c2i knockdown plants.

Supplemental Figure S8. The growth retardation phenotype of the c1c2i knockdown plants is not rescued by the high-CO2 growth condition.

Supplemental Figure S9. Reduced expression of the γCAL1 and γCAL2 genes modestly affects the maximum quantum efficiency of PSII.

Supplemental Figure S10. Normal photosynthetic apparatus of the c1c2i mutant plants.

Supplemental Figure S11. Possible biochemical functions of the γCAL proteins.

Supplemental Table S1. Normal gametogenesis of the c2iC2i2 and C1c1c2i2 hemizygotes.

Supplemental Table S2. Oligonucleotide primers used in this study.

Supplemental Materials and Methods S1.

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LITERATURE CITED


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