Chlorophyll *b* Reductase Plays an Essential Role in Maturation and Storability of Arabidopsis Seeds[1][W]

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Although seeds are a sink organ, chlorophyll synthesis and degradation occurs during embryogenesis and in a manner similar to that observed in photosynthetic leaves. Some mutants retain chlorophyll after seed maturation, and they are disturbed in seed storability. To elucidate the effects of chlorophyll retention on the seed storability of Arabidopsis (*Arabidopsis thaliana*), we examined the *non-yellow coloring1* (*nyc1*)/*nyc1-like* (*nol*) mutants that do not degrade chlorophyll properly. Approximately 10 times more chlorophyll was retained in the dry seeds of the *nyc1/nol* mutant than in the wild-type seeds. The germination rates rapidly decreased during storage, with most of the mutant seeds failing to germinate after storage for 23 months, whereas 75% of the wild-type seeds germinated after 42 months. These results indicate that chlorophyll retention in the seeds affects seed longevity. Electron microscopic studies indicated that many small oil bodies appeared in the embryonic cotyledons of the *nyc1/nol* mutant; this finding indicates that the retention of chlorophyll affects the development of organelles in embryonic cells. A sequence analysis of the *NYC1* promoter identified a potential abscisic acid (ABA)-responsive element. An electrophoretic mobility shift assay confirmed the binding of an ABA-responsive transcriptional factor to the *NYC1* promoter DNA fragment, thus suggesting that *NYC1* expression is regulated by ABA. Furthermore, *NYC1* expression was repressed in the ABA-insensitive mutants during embryogenesis. These data indicate that chlorophyll degradation is induced by ABA during seed maturation to produce storable seeds.

Chlorophyll biosynthesis is essential in the formation of photosystems. This process is most active during the development of green leaves (Ohashi et al., 1989). On the other hand, most chlorophyll molecules are degraded to safe molecules during leaf senescence (Hörtensteiner, 2006). This process is believed to be a "detoxification" process, as chlorophyll is a potential photosensitizer when it is not integrated in the photosystems (Hörtensteiner, 2004). In addition, chlorophyll degradation is also important for the allocation of carbon and nitrogen resources from senescent leaves to developing organs. The synthesis and degradation of chlorophyll also occurs in maturing seeds in a manner similar to that observed in photosynthetic leaves. In contrast to the well-examined physiological roles of chlorophyll metabolism in leaves, the physiological functions of chlorophyll metabolism are poorly understood in maturing seeds.

In maturing seeds, chlorophyll functions to sustain photosynthetic capacity (Borisjuk et al., 2004; Weber et al., 2005), although photosynthetic CO_2 fixation is low in embryos when compared with that in leaves (Asokanthan et al., 1997). Besides, photosynthesis in embryos is not even essential for seed development, as is evident from a study with chlorophyll-deficient mutants reporting that seeds of the Arabidopsis (*Arabidopsis thaliana*) *albino* mutant can germinate (Sundberg et al., 1997). Therefore, it is hypothesized that seed photosynthesis plays additional roles to simply fixing CO_2 in developing seeds (Albrecht et al., 2008). Specifically, it is suggested that the main function of photosynthesis is to increase O_2 concentration in developing seeds in order to sustain ATP synthesis in mitochondria (Baud and Lepiniec, 2010). In fact, it is reported that O_2 levels are very low in the dark and markedly increase upon illumination (Rolletschek et al., 2002). The demand for ATP synthesis is postulated to be high in oilseeds that synthesize a large amount of lipids, because this type of seed requires higher energy consumption for lipid biosynthesis compared with those for sugar or protein. This hypothesis is supported by a few experimental observations. In developing seeds of rapeseed (*Brassica napus*), the application of O_2 improved ATP levels and lipid biosynthesis to a greater extent than sugar synthesis (Vigeolas et al., 2003). In developing rapeseed embryos cultured in medium, illumination enhanced the synthesis of oil, whereas the application of a photosynthesis inhibitor reduced oil synthesis

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(Goffman et al., 2005). In addition, it is possible to assume that seed photosynthesis contributes to the activation of lipid synthesis, as acetyl-CoA carboxylase, a key enzyme of fatty acid synthesis, is activated upon reduction, and a redox potential generated by photosynthesis is involved in this activation through thioredoxin (Sasaki et al., 1997; Weber et al., 2005). Thus, it is feasible that photosynthesis serves a key role for seed development, although it is not essential as described above.

The function and regulation of chlorophyll degradation in the late phase of seed maturation are not entirely understood. It is possible that chlorophyll degradation has additional physiological functions in seeds compared with those in leaves. Chlorophyll content has been reported to be inversely related to seed quality. Specifically, when seeds contain lower amounts of chlorophyll, they show high germination rates (Jalink et al., 1998). A similar inverse relationship between seed chlorophyll contents and their germination rates has also been reported with several *abi* mutants, which are insensitive to abscisic acid (ABA). Seeds from several independent *abi* mutants fail to degrade chlorophyll during maturation and are green even after desiccation (Ooms et al., 1993; Clerkx et al., 2003; Vigeolas et al., 2003). Chlorophyll accumulation is also evident in seeds in which the ABA activity was blocked by the introduction of an antibody against the ABA in tobacco (*Nicotiana tabacum*; Phillips et al., 1997). However, as ABA is responsible for multiple processes in seed development, it is not clear whether chlorophyll retention is the major cause of the low seed germination rates in *abi* mutants. Analysis of the other class of mutants in which chlorophyll degradation is specifically impaired would be helpful to clarify the effects of chlorophyll retention on seed germination.

The first step of chlorophyll degradation is the conversion of chlorophyll *b* to chlorophyll *a* by the chlorophyll cycle (Fig. 1; Hörtensteiner, 2006; Tanaka and Tanaka, 2006, 2007). Chlorophyll *a* is then converted to pheophytin by an unidentified magnesium dechelatase. The phytol tail of pheophytin is removed by pheophytinase (Schelbert et al., 2009), and this step is followed by an oxidative ring opening by pheophorbide *a* oxygenase (PaO; Pruzinská et al., 2003; Tanaka et al., 2003). Mutations in these chlorophyll degradation enzymes result in the stay-green phenotype. However, some chlorophyll degradation mutants exhibit not only the stay-green phenotype but also other physiological symptoms. For example, the *pao* mutant, which has a defect in PaO and accumulates pheophorbide *a*, displays both the stay-green (Pruzinská et al., 2003) and severe cell-death (Tanaka et al., 2003) phenotypes even under complete darkness (Hirashima et al., 2009). Chlorophyll *b* reductase catalyzes the conversion of chlorophyll *b* to 7-hydroxymethyl chlorophyll *a*, which is the first step in chlorophyll *b* degradation (Ito et al., 1996; Rüdiger, 2002; Kusaba et al., 2007). Mutants lacking chlorophyll *b* reductase show a stay-green phenotype in leaves. This mutant could possibly serve as a valuable tool for elucidating the relationship between chlorophyll retention and storability, because it does not show cell-death or other severe phenotypes.

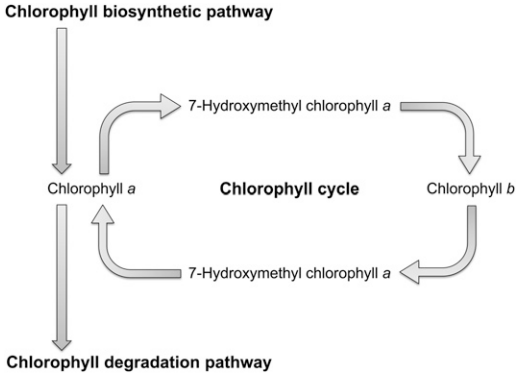

Figure 1. Chlorophyll metabolism pathway. Chlorophyll metabolism can be classified into three pathways. The first pathway of chlorophyll metabolism encompasses the synthesis of chlorophyll *a* from Glu. The second pathway, which is termed the chlorophyll cycle, includes the interconversion of chlorophyll *a* and chlorophyll *b*. The final pathway involves the degradation of chlorophyll *a*. In the chlorophyll cycle, chlorophyll *b* is degraded after conversion to chlorophyll *a*. Chlorophyll *b* reductase catalyzes the conversion of chlorophyll *b* to 7-hydroxymethyl chlorophyll *a*.

Two chlorophyll *b* reductase isoforms, Non-Yellow Coloring1 (NYC1)-Like (NOL) and NYC1, were identified in both rice (*Oryza sativa*; Sato et al., 2009) and Arabidopsis (Horie et al., 2009). NOL and NYC1 are colocalized in the thylakoid membrane and function in a complex as a heteromeric chlorophyll *b* reductase enzyme in rice. As a result, a mutation in either *NOL* or *NYC1* results in a stay-green phenotype (Sato et al., 2009). In contrast, *NOL* and *NYC1* are differentially expressed in Arabidopsis during development, and only a *nyc1* mutation was accompanied by a stay-green phenotype (Horie et al., 2009). Thus, it is likely that these genes have distinct and independent functions in Arabidopsis.

In this study, we investigated the impact of chlorophyll retention on embryogenesis and seed storability using the Arabidopsis *nol* and *nyc1* mutants. Relative to the wild type, a large amount of chlorophyll was retained in the *nyc1* mutant seeds. The relationship between *NYC1* expression and chlorophyll retention was also supported by analysis of the *abi3* mutant. Soon after harvest, approximately 75% of *nyc1/nol* mutant seeds were able to germinate, but the seed viability of the mutants was drastically reduced as a result of prolonged storage. Using electron microscopy, we demonstrated that oil bodies (OBs) are reduced in size in the mutant seeds. These studies demonstrate that chlorophyll degradation is essential for maintaining the viability of seeds during storage.

RESULTS

Retention of Chlorophyll in Seeds

To examine the possible involvement of NYC1 and NOL in chlorophyll degradation during seed maturation, we analyzed the chlorophyll content in dry seeds of wild-type and mutants plants (Table I). In the wild type and the *nol* mutant, only a small amount of chlorophyll remained in the dry seed, and the chlorophyll *a/b* ratios were 1.70 and 2.35, respectively. In contrast, a large amount of chlorophyll remained in the *nyc1* and *nyc1/nol* mutants, and the chlorophyll *a/b* ratios of these mutants were below 1.0. The wild type and the *nol* mutant had similar chlorophyll levels after seed maturation, indicating that the mutation in the *NOL* gene was almost completely compensated by the *NYC1* gene. In contrast, the chlorophyll content of the *nyc1* mutant was significantly higher than in the wild type but lower than in the *nyc1/nol* mutant, suggesting that NOL also participates in chlorophyll degradation during seed maturation. These results indicate that NYC1 plays a major role in chlorophyll degradation during seed maturation, and the contribution of NOL to this process seems less than that of NYC1.

In leaves, chlorophyll molecules are always associated with chlorophyll-binding proteins. In particular, chlorophyll *a* is associated with the core complexes and light-harvesting chlorophyll *a/b*-binding protein complex (LHC) proteins of photosystems, while chlorophyll *b* is associated with only LHC proteins. It is reported that a lack of chlorophyll *b* reductase results in the retention of LHC proteins as well as both chlorophyll *a* and *b* that are associated with LHC proteins in leaves (Kusaba et al., 2007). It is hypothesized that the *nyc1/nol* seeds also retain LHC proteins, but they might not retain the protein subunits of the core complexes. To verify this hypothesis, we examined the level of chlorophyll-binding proteins in dry seeds (Fig. 2) by immunoblotting. Small amounts of the LHCII apoproteins accumulated in wild-type and *nol* mutant seeds, but a large amount of LHCII apoproteins accumulated in the *nyc1* and *nyc1/nol* mutants. It is not clear at present in which cellular localization LHCII accumulated. In leaves of the *nyc1/nol* mutant, LHCII exists in thylakoid membranes of senescent chloroplasts. In seed cells, these chloroplasts should be transformed to another type of plastids (e.g. leucoplasts). It would be reasonable to assume that LHCII is retained in these plastids. Specifically, LHCII might be localized in the remnant of thylakoid membranes in these plastids. Although it is not obvious from our electron microscopic observations, it is possible that embryos contain a membrane structure similar to a premature form of thylakoid membranes, a form of which might be similar to the prothylakoid membranes in etiolated cotyledons. In both wild-type and mutant seeds, the levels of the subunits of the core complexes (PsaA/B and D1) were beneath the detectable limits by immunoblotting (Fig. 2). These observations indicate that chlorophyll degradation proceeds in the same manner in both the developing embryo and the senescent leaf.

Table I. *Chlorophyll contents in wild-type, nyc1 mutant, nol mutant, nyc1/nol mutant, and abi3 mutant seeds*

Fifty seeds were absorbed in water overnight. The water-saturated seeds were ground in acetone. Extracted chlorophyll was analyzed by HPLC. SD values are indicated for three samples.

Plants	Chlorophyll Content		Chlorophyll *a/b* Ratio
	Chlorophyll *a*	Chlorophyll *b*	
	pmol seed^{-1}		
Wild type	0.29 ± 0.041	0.17 ± 0.042	1.70 ± 0.156
nyc1	0.76 ± 0.080	1.05 ± 0.104	0.73 ± 0.038
nol	0.16 ± 0.019	0.07 ± 0.014	2.35 ± 0.239
nyc1/nol	2.17 ± 0.283	4.14 ± 0.582	0.53 ± 0.006
abi3	1.00 ± 0.096	0.65 ± 0.096	1.55 ± 0.153

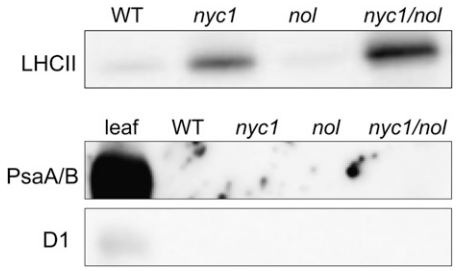

Figure 2. Immunoblot analysis of LHCII, PsaA/B, and D1 in wild-type (WT), *nyc1* mutant, *nol* mutant, and *nyc1/nol* mutant seeds. Fifty water-saturated seeds were homogenized with 200 μL of extraction buffer, and 5 μL of the extract was subjected to SDS-PAGE. Immunoblot analysis was subsequently performed to detect LHCII, PsaA/B, and D1. Leaf extraction was used for the positive control for PsaA/B and D1 detection.

Chlorophyll Content during the Early Germination Phase

To examine the chlorophyll retention in germinating seeds, we used fluorescence microscopy to measure the fluorescence of the *nyc1/nol* mutant seeds. The dry seeds were imbibed for 2 d at 4°C, and the fluorescence was subsequently observed. Most of the wild-type seeds exhibited low fluorescence, thus indicating that the wild type contains a low level of chlorophyll in seeds (Fig. 3B). In contrast, the intensities of fluorescence varied among the mutant seeds, with some seeds exhibiting high fluorescence and others showing low fluorescence (Fig. 3E). The variation of chlorophyll retention in the mutant seeds was reported with the SnRK2 mutant producing green seeds (Nakashima et al., 2009). In this study, a subsequent evaluation of the chlorophyll content using HPLC revealed a direct relationship between the chlorophyll content and the chlorophyll fluorescence in seeds. It should be noted that the size of the seeds did not differ between the wild type and the *nyc1/nol* mutant (Fig. 3, A and D).

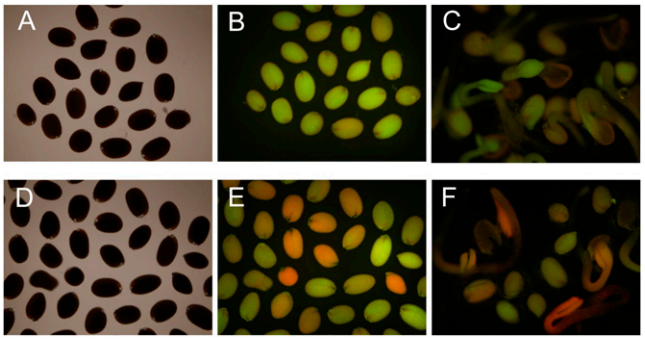

Figure 3. Observation of wild-type and *nyc1/nol* mutant seeds using transmission and fluorescence microscopes. Wild-type seeds and *nyc1/nol* mutant seeds were sown for 1 d. Fluorescence was observed by exciting the samples with a blue light source. A to C, Wild-type seeds. D to F, *nyc1/nol* mutant seeds. A and D show light microscopy, and B and E show florescence microscopy. Seedlings were grown for 1 d under dark conditions at 23°C and observed by fluorescence microscopy (C and F).

To examine the localization of chlorophyll, the seeds were germinated for 1 d in dark conditions at 20°C after 2 d of imbibition at 4°C. The roots appeared from the seed coats and the hypocotyl elongated, but some cotyledons were still covered by the seed coats (Fig. 3, C and F). In the wild-type seedlings, the levels of fluorescence in the hypocotyls and cotyledons were very low (Fig. 3C). In contrast, the hypocotyl and the cotyledon showed strong fluorescence in the *nyc1/nol* mutant (Fig. 3F), indicating that the observed fluorescence of the intact seeds (Fig. 3E) was derived from chlorophyll in the hypocotyl and the cotyledons. Next, we determined chlorophyll levels of fluorescent and nonfluorescent seeds stored for 14 months by HPLC. The fluorescent seeds contained 2.95 ± 1.62 pmol chlorophyll seed^{-1}, whereas nonfluorescent seeds contains only 0.75 ± 0.11 pmol chlorophyll seed^{-1}, indicating that high-fluorescence seeds actually accumulated high levels of chlorophyll. In order to confirm the localization of chlorophyll in seeds, we measured the fluorescence spectra using confocal microscopy. After imbibition for 2 d at 4°C in the dark, the seeds were gently pinched by cover grass to detach the embryo from the seed coats (Fig. 4A); the cotyledons were revealed by this treatment. We then quantified the fluorescence spectra of specific areas of tissues, as indicated using circles and squares. Figure 4B shows the fluorescence intensity per area at various wavelengths. In the wild-type seeds, the cotyledons exhibited a fluorescence maximum at approximately 680 nm, thus indicating chlorophyll fluorescence. In contrast, chlorophyll fluorescence was not observed in the seed coats. High levels of chlorophyll fluorescence were observed in the mutant embryos. In contrast, the fluorescence spectrum of the mutant seed coat did not show a peak at around 680 nm, which is a hallmark of the spectrum of in vivo chlorophyll fluorescence. These results indicate that seed coats of the mutant do not accumulate chlorophyll and that the chlorophyll found in the dry seeds of the *nyc1/nol* mutant (Table I) accumulated in the embryo.

Storability of Chlorophyll-Containing Seeds

Chlorophyll retention was sometimes accompanied by low storability (Parcy et al., 1997). To address the question of whether chlorophyll retention is correlated to the storability of seeds, we performed a germination assay after storage for various periods at room temperature (Fig. 5A). Almost all of the wild-type seeds germinated immediately after seed maturation, and only a slight decrease in the germination rate was observed, even after storage for a prolonged period of 42 months. In contrast, approximately 75% of the *nyc1/nol* mutant seeds geminated without storage. However, in the case of the mutant, the germination rates decreased markedly as a result of storage, and most seeds failed to germinate after a prolonged period of storage for a total of 23 months. Based on these observations, we concluded that chlorophyll degradation is essential for seed storability.

When the seeds were germinated under light conditions, chlorophyll synthesis initiated and the cotyledons accumulated chlorophyll and turned green

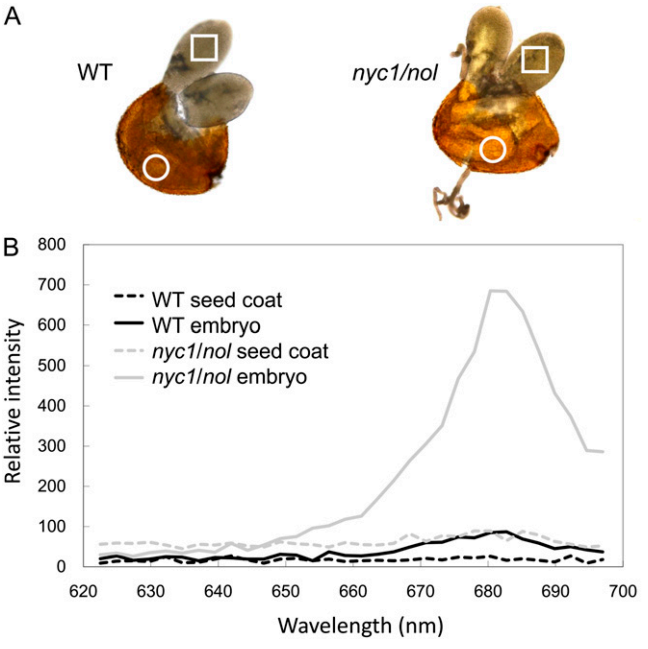

Figure 4. Fluorescence spectrum analysis of wild-type seeds (WT) and *nyc1/nol* mutant seeds. A, Transmission microscopy of wild-type seeds and *nyc1/nol* mutant seeds. Wild-type seeds and *nyc1/nol* mutant seeds were imbibed in water. The seeds were then broken softly to detach embryos from the seed coats. Squares show embryo, and circles show seed coat. B, Fluorescence spectrum. Chlorophyll fluorescence was detected using a confocal laser scanning microscope with an excitation wavelength set at 408 nm.

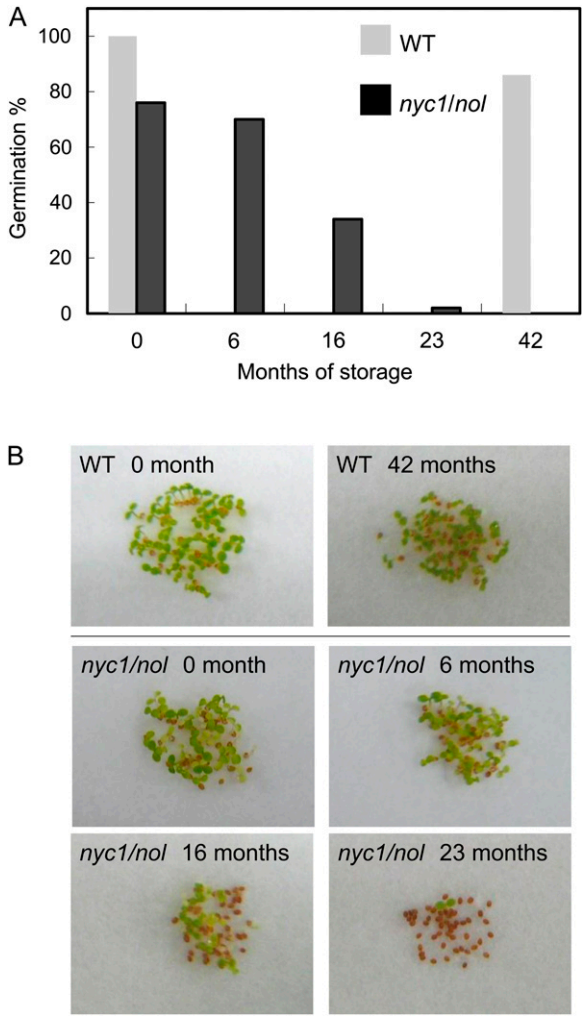

Figure 5. Germination behavior of seeds. A, Germination rate changes during the storage of seeds. Germination behavior was tested at several time points during the prolonged storage of wild-type seeds (WT) and *nyc1/nol* mutant seeds. Wild-type seeds and *nyc1/nol* mutant seeds were sown on filter paper at 4°C for 2 d and then placed at 23°C under light conditions for 4 d, and the number of germinated seedlings was measured. B, Effect of storage time on the phenotypes of germinating seedlings. Seedlings were grown as described for A.

(Fig. 5B). All of the cotyledons in the wild-type seedlings became uniformly green, thus indicating that the chlorophyll synthesis rates were similar among individual seedlings. In contrast, the chlorophyll accumulation exhibited a high level of plant-to-plant variation in the mutants, which can be classified into three groups. The first type of cotyledons exhibited an accumulation of chlorophyll and green coloration similar to the wild-type seeds. The second mutant phenotype of the seeds was characterized by a pale green coloration and a reduction of chlorophyll accumulation. In the third mutant phenotype, the cotyledons were completely white and lacked chlorophyll accumulation. In spite of the difference in the color of the cotyledons, the rosette leaves exhibited similar levels of chlorophyll accumulation and a resultant green coloration (Supplemental Fig. S1). Considering that cotyledons are developed during seed formation, and that rosette leaves are the result of vegetative growth after germination, it is plausible that the pale green and white cotyledons might result from the retention of chlorophyll during seed maturation.

Figure 6 shows the changes in chlorophyll content that resulted from prolonged seed storage. Specifically, both the wild type and the *nyc1/nol* mutant accumulated chlorophyll in their seeds before storage. However, the levels of chlorophyll were reduced as a consequence of the prolonged storage of seeds. These observations suggest that chlorophyll degradation associates with a defect in germination in the *nyc1/nol* mutant. The fresh mutant seeds were classified into two groups: one group exhibited fluorescence and the other group did not (Fig. 7A). The germination rate of the seeds exhibiting fluorescence was high; in contrast, most of the seeds exhibiting no fluorescence did not germinate (Fig. 7, B and C).

Intracellular Structure of Embryonic Cotyledons

It is expected that storability depends on the integrity of the inner structure of the mature seeds. The inner structures of the wild-type and mutant seeds were investigated using electron microscopy (Fig. 8). No distinct differences in the cell sizes were detected for the embryonic cotyledons between the wild type and the *nyc1/nol* mutant, an observation that is consistent with the similarity of their seed sizes (Fig. 3). In contrast, there were alterations detected for the various intracellular structures in the mutant seeds. Some

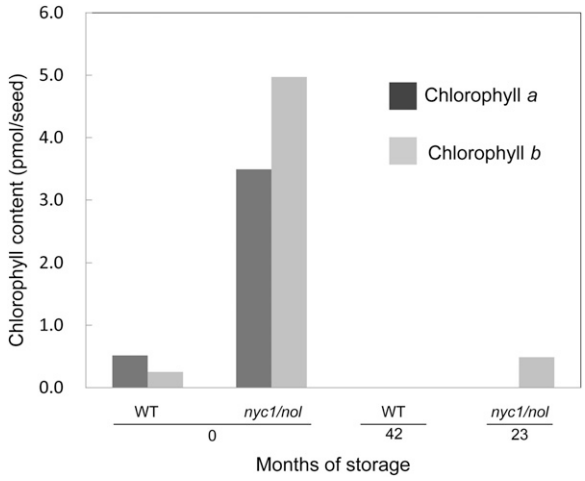

Figure 6. Analysis of chlorophyll *a* and chlorophyll *b* contents of wild-type seeds (WT) and *nyc1/nol* mutant seeds. After 0, 42 (wild type), and 23 (*nyc1/nol* mutant) months of storage, the wild-type seeds and *nyc1/nol* mutant seeds were homogenized in acetone. The extracted chlorophyll contents were measured.

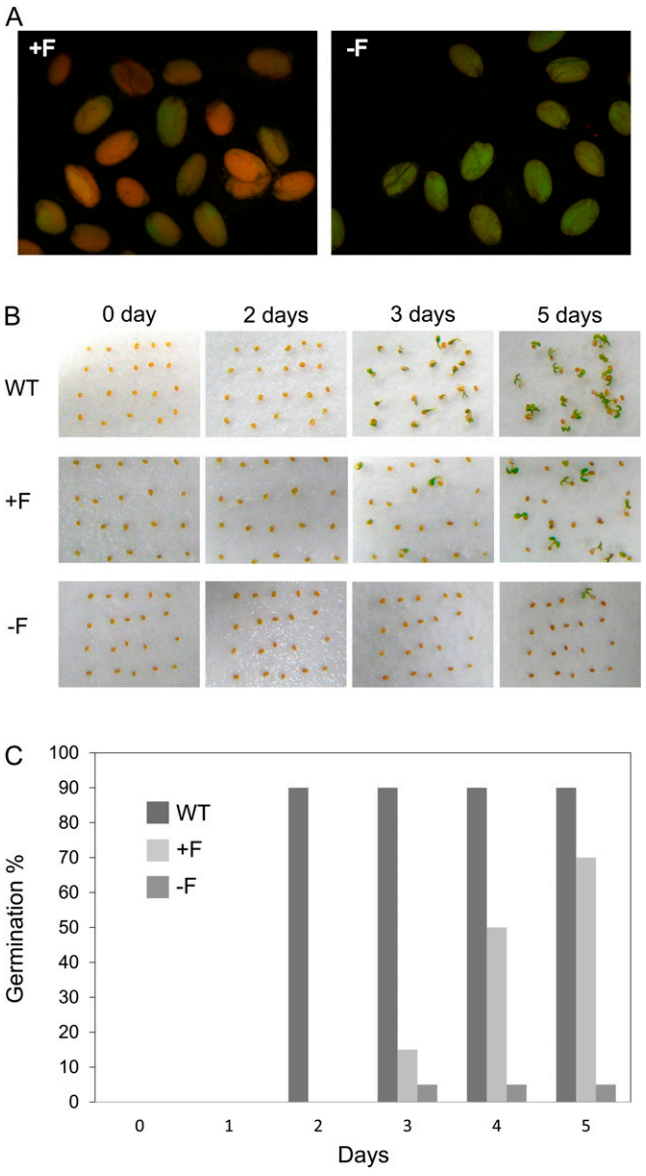

Figure 7. Germination behavior of *nyc1/nol* mutants. A, Observation of *nyc1/nol* mutant seeds using a fluorescence microscope. The seeds were sown on filter paper at 4°C for 3 d and separated into fluorescent seeds (+F) and nonfluorescent seeds (−F). Fluorescence was observed by exciting the samples with a blue light source. B, Germination behavior of seeds. Wild-type seeds (WT) and *nyc1/nol* mutant seeds with or without fluorescence were sown on filter paper at 4°C for 2 d and then placed at 23°C under light conditions for 5 d. Seedlings were observed at several time points. C, Germination rates of wild-type seeds and *nyc1/nol* mutant seeds. Germination rates were calculated from B.

mutant cells contained normal-sized OBs (Fig. 8, C and E), but other cells contained a large number of small OBs (Fig. 8, D and F). In contrast, no wild-type seed cells contained such a large number of small OBs (Fig. 8, A and B). Storage also had no significant effect on the cellular structure of both wild-type (Fig. 8, A and B) and mutant (Fig. 8, C–F) seeds.

NYC1 and *NOL* Promoter-GUS Analysis

Chlorophyll retention was observed in the seeds from both the *nyc1* and *nyc1/nol* mutants, and *NOL* partly rescued the *nyc1* phenotype (Table I). These data suggest that both NYC1 and NOL proteins participate in chlorophyll degradation during seed maturation. In the next level of investigation, we examined the expression patterns of the *NYC1* and *NOL* genes by monitoring the activity of GUS, which was expressed under the control of the *NYC1* and *NOL* promoter regions, respectively (Fig. 9). GUS staining was not observed in green leaves of the transformant containing the *NYC1* promoter-GUS construct. These data are consistent with the observation that the *NYC1* gene is only expressed in senescent Arabidopsis leaves (Horie et al., 2009). In contrast, GUS staining was observed in embryos in their late developmental phase in the transformants, which express the *GUS* gene under the control of the *NYC1* promoter. Likewise, the transformant containing the *NOL* promoter-GUS construct also showed expression of the *GUS* gene in embryos. The only difference we observed with the transformants for the *NYC1* promoter-*GUS* and the *NOL* promoter-*GUS* constructs was that weak GUS staining was observed with the green leaves in the *NOL* promoter-*GUS* transformant. These results were consistent with the data on *NYC1* and *NOL* expression from the genome-wide analysis (Xiang et al., 2011), which confirm that both *NYC1* and *NOL* are expressed in maturing seeds. Collectively, these results support

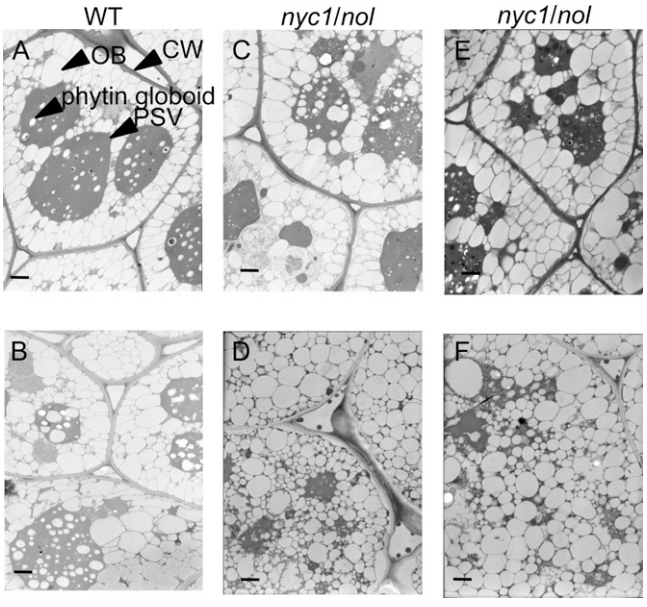

Figure 8. Transmission electron micrographs of wild-type and *nyc1/nol* mutant seeds. Wild-type seeds (WT) and *nyc1/nol* mutant seeds were sown for 1 d and observed. Phytin globoid cavities are observed as the small white circular areas. CW, Cell wall; PSV, protein storage vacuole. A, Wild-type seed, stored for 13 months. B, Wild-type seed, stored for 72 months. C and D, *nyc1/nol* seeds, stored for 10 months. E and F, *nyc1/nol* seeds, stored for 54 months. Bars = 1 μm.

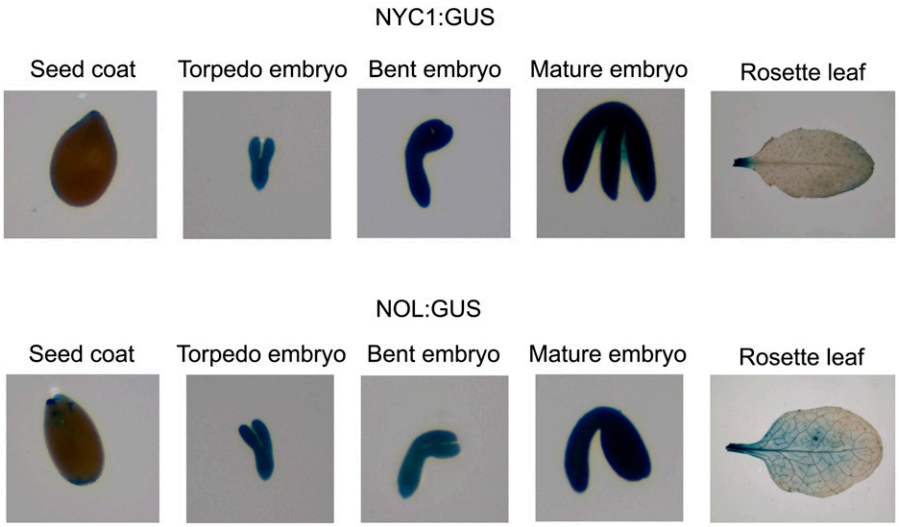

Figure 9. Histochemical staining of GUS activity of the *NYC1* promoter-GUS and *NOL* promoter-GUS transformants. The 2-kb *NYC1* promoter region and *NOL* promoter region were fused to GUS and introduced into wild-type plants. The transformants were grown under standard conditions. Seed coats, embryos in maturing seeds, and rosette leaves were subsequently stained for spatial observation of the *NYC1* and *NOL* expression patterns.

the idea that NYC1 is involved in the degradation of chlorophyll during seed maturation and that NOL partly contributes to chlorophyll degradation. It should be noted that these genes were not expressed in seed coats (Fig. 9), which is consistent with the absence of chlorophyll in the seed coat.

Electrophoretic Mobility Shift Assay

ABA is one of the key plant hormones that functions in the seed maturation process. As mentioned in the introduction, ABA-insensitive mutants (*abi*) are characterized by a retention of chlorophyll and a consequent green coloration of their mature seeds. This phenomenon suggests that chlorophyll degradation is controlled by ABA in the seeds. A sequence analysis of the *NYC1* promoter identified a potential ABA-responsive element (ABRE) containing an ACGT motif at −284 from the translation initiation start site (Fig. 10A). This element is known to interact with transcription factors from the bZIP family. Because we hypothesized that *NYC1* expression is induced by the binding of an ABRE-binding protein to the putative ABRE in seeds, we used the electrophoretic mobility shift assay (EMSA) to test the ability of ABF4, which is an ABRE-binding factor, to drive *NYC1* expression.

Using a yeast one-hybrid system, ABF4 was previously identified as the ABRE-binding protein (Choi et al., 2000; Uno et al., 2000). Recombinant ABF4 protein (alternatively termed AREB2) was tested for its binding activity to the putative ABRE within the *NYC1* promoter by EMSA. This protein is readily abundant in the early phase of seed maturation (Ruuska et al., 2002). We used a truncated form of the ABF4 protein that consists of the bZIP domain (amino acids 331–440) because previous reports indicated the difficulties in expressing full-length ABF4 (Comelli et al., 2009). Biotin-labeled DNA nucleotides, comprising −294 to −234 of the *NYC1* promoter, were tested for interaction with the truncated form of the ABF4 protein. A mobility shift of the labeled DNA was clearly observed in the presence of recombinant ABF4 in an EMSA (Fig. 10B). In contrast, when the recombinant ABF4 protein was withheld from the reaction mixture, the shift was not observed. To provide further evidence that the ABF4 protein drives *NYC1* gene expression, we mutated several nucleotides in the G-box-binding region of the *NYC1* promoter. These mutations abolished the ability of ABF4 to bind to the DNA fragment, and no shifting was observed. These data clearly indicate that the binding of ABF4 to the *NYC1* promoter fragment requires the integrity of the G-box. Considering that several bZIP proteins belonging to the ABI5/ABF/AREB family are active during seed maturation (Ruuska et al., 2002), it is likely that *NYC1* expression is regulated by ABA through the binding of a bZIP transcription factor to its promoter region.

Expression of *NYC1* during Embryo Development

abi3 mutants derived from the Landsberg *erecta* ecotype produce green seeds (Parcy et al., 1997). We determined the chlorophyll level in the *abi3* mutant of the Columbia ecotype seeds (Table I). The chlorophyll content in the *abi3* mutant seeds was 1.65 pmol seed^{-1}, which was higher than that of wild-type seeds (0.46 pmol seed^{-1}). These observations suggest that ABA regulates the expression of *NYC1* during embryo development. To confirm the regulation of *NYC1* expression by ABA, the expression of *NYC1* in the embryo of the *abi3* mutant was determined. mRNA was extracted from both the maturing seeds of the wild type and the mutant. The expression of *NYC1* mRNA was determined by a semiquantitative reverse transcription (RT)-PCR analysis (Fig. 11). Lower levels of the *NYC1* transcript were detected in the *abi3* mutant compared with the wild type. These observations suggest that during maturation of seeds, ABA induces

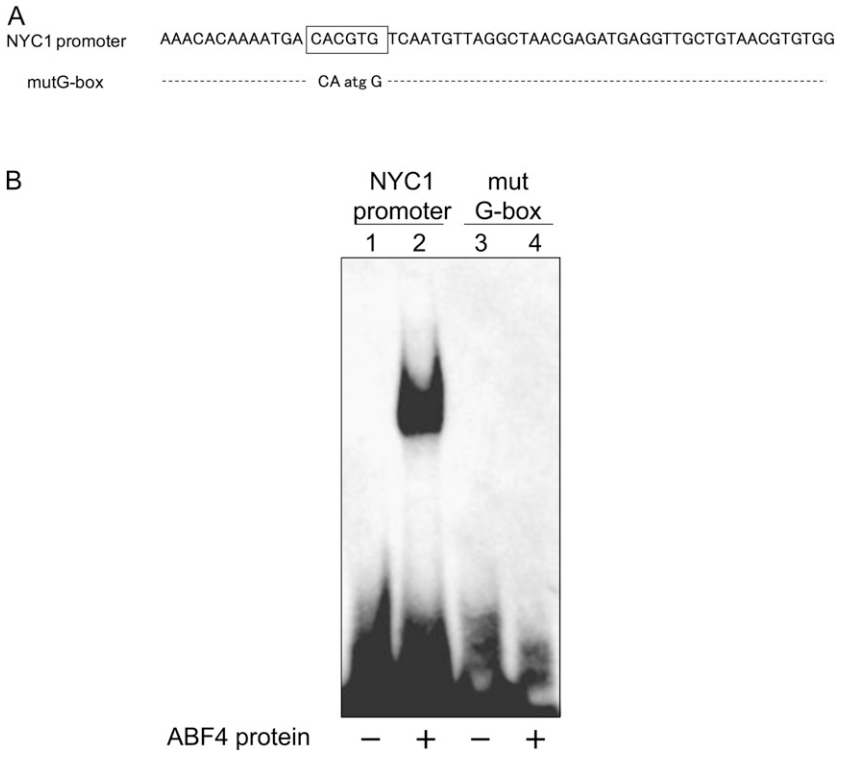

Figure 10. Binding assay of ABF4 to the *NYC1* promoter. A, *NYC1* promoter sequence. Nucleotides −294 to −234 are shown, and the G-box is boxed. Nucleotides altered by mutagenesis are shown in lowercase letters. B, EMSA analysis of ABF4 and the *NYC1* promoter. The binding assay was performed using either a biotin-labeled *NYC1* promoter fragment (lanes 1 and 2) or a similar fragment in which the G-box was mutagenized (mut G-box; lanes 3 and 4).

NYC1, and the chlorophyll degradation proceeds; however, in the *abi3* mutant, *NYC1* expression is suppressed because of its insensitivity to ABA, and the chlorophyll degradation is defective.

DISCUSSION

Chlorophyll Accumulation in Mutant Seeds

Fluorescence microscopic observations of the early-germinating seeds indicated that chlorophyll mainly accumulates in the cotyledons and hypocotyls, and it accumulated at very low levels in the seed coats (Fig. 4). The NYC1 and NOL proteins do not have a remarkable effect on chlorophyll synthesis in leaves, because the chlorophyll levels of the mutant and wild-type leaves are nearly equivalent (Horie et al., 2009). Therefore, it is reasonable to hypothesize that chlorophyll is synthesized normally during seed development, but chlorophyll degradation is specifically inhibited during seed maturation in mutants. Considering that LHCII apoproteins were still detected in mutant seeds but other chlorophyll binding proteins were not present (Fig. 2), it is possible that chlorophyll binds to LHCII apoproteins in dry seeds. These results in the seeds were consistent with those observed in senescent leaves of the chlorophyll *b* reductase mutant, in which only LHCII remained in the mutant, while other chlorophyll-binding proteins completely disappeared (Kusaba et al., 2007; Horie et al., 2009). It is possible that the degradation of chlorophyll during seed maturation proceeds in a similar manner to how it proceeds in senescent leaves.

Regulation of NYC1 Expression in Seed Development

The accumulation of chlorophyll in *nyc1* mutant seeds indicates the crucial role of NYC1 in the degradation of chlorophyll during seed maturation. This scenario is consistent with the GUS-staining activity of the *NYC1* promoter that enabled us to characterize *NYC1* expression in embryos. These data were also supported by a recent study showing that, in addition to *Pheophytinase* and *PaO*, *NYC1* is expressed in the late stages of seed development (Xiang et al., 2011). We concluded that NYC1 is essential for the chlorophyll degradation observed during seed maturation.

In Arabidopsis, many reports have elucidated a functional role of ABA in the process of chlorophyll degradation in seeds. ABI3 is a transcription factor that controls ABA-induced seed maturation (Nambara and Marion-Poll, 2003). In *abi3* mutants, the chlorophyll breakdown is reduced at the end of seed maturation, thus resulting in the typical green-seed phenotype. When biologically available levels of ABA are reduced by immunomodulation in embryos, the chlorophyll degradation is suppressed in mature seeds (Phillips et al., 1997). These observations indicate that one of the functions of ABA during seed maturation is to induce the degradation of chlorophyll. In rice leaves, ABA treatment induced *NYC1* expression (Kusaba et al., 2007). Additionally, the expression of *NYC1*, but not *NOL*, was induced by ABA treatment in Arabidopsis seedlings (Matsui et al., 2008). However, the regulation of chlorophyll degradation enzymes by ABA during seed maturation has not been reported previously. Many ABA-inducible genes contain ABRE

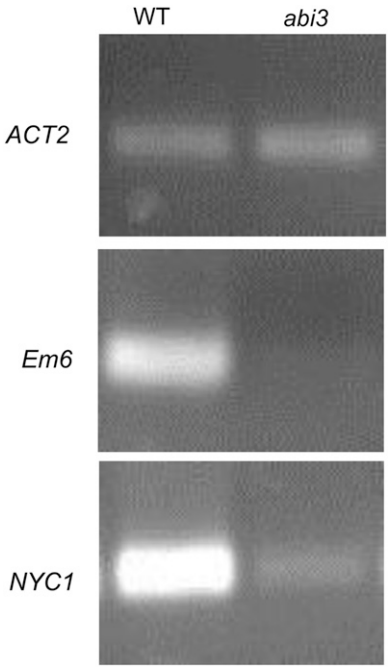

Figure 11. Semiquantitative RT-PCR analysis of gene expression in wild-type seeds (WT) and *abi3* mutant seeds. RNA was isolated from maturing seeds in the pods. The numbers of PCR cycles are 29 (*ACT2*), 25 (*Em6*), and 26 (*NYC1*). *ACT2* was used as a control for ubiquitous expression, and *Em6* was used as a control for ABA-induced expression.

(PyACGTGGC) in their promoter regions (Nambara and Marion-Poll, 2003). The ABRE region functions as a cis-acting element involved in ABA-regulated gene expression. The ABRE region was first identified in the wheat (*Triticum aestivum*) *Em* gene functioning in seeds during embryogenesis (Guiltinan et al., 1990). A putative ABRE region (CACGTGTC) was found in the *NYC1* promoter, and the interaction of ABRE with the ABF4 bZIP transcription factor was experimentally tested using EMSA (Fig. 10). The expression of *NYC1* in the maturing seeds was suppressed in the *abi3* mutant (Fig. 11), thus indicating that chlorophyll degradation is at least partly controlled by ABA through the regulation of *NYC1* expression. This finding is consistent with the claim that the genes responsible for chlorophyll degradation, *Pheophytinase*, *PaO*, and *SGR*, are expressed in the embryo (Xiang et al., 2011) and that their transcription levels are regulated by ABA in seedlings (Matsui et al., 2008).

Chlorophyll Degradation in Seeds Is Important for Seed Maturation and Storage

In an effort to conserve resources in whole plants, the senescent leaves finally die after nitrogen and carbon resources are reallocated to other sink tissues. However, because the seed cells must survive, chlorophyll degradation in the seeds should be more tightly regulated, because chlorophyll and its degradation intermediates are highly reactive and potent molecules. As mentioned in the introduction, many reports have described the close relationship between chlorophyll retention and germination rates. However, in the context of germinating seeds, the exact effects of chlorophyll retention are still poorly understood. The chlorophyll *b* reductase mutant has served as a useful genetic tool to elucidate the functional relationship between chlorophyll retention and seed quality.

When the mutant seeds were analyzed, the sizes of the OBs were small. The chloroplasts in the senescent leaves of the chlorophyll *b* reductase mutants do not properly convert to gerontoplasts, thus resulting in the retention of a large size of grana stacks. Similar observations were also reported in a soybean (*Glycine max*) mutant. Specifically, the soybean chlorophyll-retention mutant *cytG* has a much higher level of chlorophyll *b* and elevated chlorophyll *a* in leaves and seeds (Guiamét et al., 1991; Chao et al., 1995). In seeds of the *cytG* mutant, stacked thylakoid membranes were detected (Chao et al., 1995). It is possible that chloroplasts are not fully converted to gerontoplasts due to the retention of LHCII and grana membranes. One possible reason for the different sizes of the OBs is that, due to the structural hindrance, the retention of the chloroplast interferes with the organization of the OBs, although the chloroplast was not found in the mature seed. Another possible reason is the incomplete production of fatty acids by the plastids. Fatty acids are produced in the plastid compartment of plant cells (Chapman and Ohlrogge, 2012). If the synthesis of the fatty acids was modified in the chlorophyll-containing plastids, then the development of OBs would be affected. However, we could not find a difference in the protein level and fatty acid level in the mature mutant seeds (Supplemental Fig. S2). Thus, the effects of chlorophyll retention on the inner structure of the seeds require further research.

Another effect of the retention of chlorophyll is a defect in seed storability. The germination of the mutant seeds rapidly decreased during storage. It is well known that seed storability is affected by many factors, such as protein denaturation (Rajjou and Debeaujon, 2008), heat shock proteins (Prieto-Dapena et al., 2006), repair enzymes for abnormal amino acid residues (Ogé et al., 2008), and antioxidant molecules (Sattler et al., 2004). The reduction of the storability of *nyc1/nol* mutant seeds might not be caused by these factors, because this mutation specifically impacts chlorophyll degradation, and it has not been functionally linked to any of the aforementioned factors. One might speculate that the chlorophyll in seeds generates reactive oxygen species that might result in the destruction of cellular components during storage. However, this is not a plausible explanation for the results observed in our experiments, because the *nyc1/nol* seeds were stored in dark conditions. As a result, there is a minimal chance for reactive oxygen species to be generated. These observations were

consistent with a report on the *abi3* mutant that described its germination rates as being similar under both light and dark conditions (Clerkx et al., 2003).

There are two possible explanations for the reduced germination rates of the *nyc1/nol* mutant. The first explanation is that seed maturation was not completed properly by the retention of chlorophyll in the mutant, thus resulting in the abnormally developed intracellular structures in the mutant (Fig. 8). However, this hypothesis is not plausible, because alteration of the subcellular structure of the mutant does not greatly affect the germination of the fresh seeds (Fig. 8). The other explanation is that chlorophyll *b* was not properly degraded in the *nyc1* mutant and that the unphysiological degradation products of chlorophyll in the *nyc1* mutant lowered the germination rate. This idea is supported by the observation that the mutant seeds with low chlorophyll levels had low germination rates (Fig. 7). The chlorophyll molecules of these seeds might have been degraded before the germination experiments. It has been suggested that chlorophyll is degraded by lipoxygenase present in the mature seeds (Klein et al., 1984; Cohen et al., 1985). It is possible that lipoxygenase activities in seeds generate toxic chlorophyll breakdown products. Chlorophyll content was high in the embryo and very low level in the seed coat (Fig. 4), indicating that chlorophyll in the embryo affects seed storability. However, it cannot be excluded that the seed coat is involved in reduction of the germination rate of the *nyc1/nol* mutant, because the seed coat controls seed germination in Arabidopsis (Lee et al., 2010).

Recent advances in research pertaining to chlorophyll metabolism has revealed that it has many physiological functions, including cell death, communication between the chloroplasts and nuclei, acclimation to light intensity, and senescence (Tanaka and Tanaka, 2006, 2007). In this study, we found that the degradation of chlorophyll in the late phase of seed maturation is functionally important to ensure the proper development of seeds. The chlorophyll content in dry seeds was not affected by a mutation of NOL, but the *nyc1* mutant contains a large amount of chlorophyll in the seeds. These data indicate that NYC1 plays a central role in chlorophyll degradation during seed maturation.

A short sublethal freezing stress causes the retention of chlorophyll in mature oilseed rapeseeds. This so-called green-seed problem is one of the major concerns of the canola industry, because the chlorophyll content of the oil reduces the overall quality of the oil (Hörtensteiner and Kräutler, 2000). PaO is known to be affected by freezing (Chung et al., 2006), and the decrease of PaO by freezing exposure is believed to interfere with the programmed degreening of the canola seed. The green-seed problem is an economically and agriculturally important issue. Elucidation of the role of chlorophyll *b* reductase and other chlorophyll degradation enzymes during seed maturation will greatly increase our ability to find means to circumvent the agriculturally important green-seed problem.

CONCLUSION

In this study, the effect of chlorophyll retention on seed storability was examined by using *nyc1/nol* mutants that do not degrade chlorophyll properly. The germination rates of chlorophyll-retaining seeds rapidly decreased during storage, indicating that chlorophyll in the seeds affects seed longevity. The retention of chlorophyll also affects the development of OBs. Expression of the chlorophyll degradation enzyme NYC1 was shown to be regulated by ABA in seeds. These studies indicate the importance of chlorophyll degradation during seed maturation and could contribute to agricultural applications.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana* Columbia ecotype) was grown at 23°C under continuous light in a growth chamber equipped with white fluorescent lamps at a light intensity of 80 μmol m^{-2} s^{-1}. The seeds were harvested from mature dry siliques and stored in darkness at room temperature.

The transferred DNA insertion mutants lacking either AT4G13250 (SALK_091664; NYC1) or AT3G24650 (SALK_138922; ABI3 [Liu et al., 2008]) were obtained from the Arabidopsis Biological Resource Center. The transferred DNA insertion mutants lacking AT5G04900 (AL759262; NOL) were obtained from GABI-Kat. The *nyc1* and *nol* mutants were crossed, and the double mutant was identified by PCR-based genotyping (Horie et al., 2009).

HPLC Analysis of Chlorophyll

The *nyc1*, *nol*, *nyc1/nol*, and *abi3* mutants and wild-type samples were harvested as mature dry seeds. Fifty seeds were imbibed in water overnight, and the water-saturated seeds were ground in 200 μL of acetone. The homogenates were centrifuged at 15,000*g* for 10 min at 4°C. Pigments were applied on a reverse-phase column, Symmetry C8 (150 × 4.6 mm; Waters), and separated with methanol. Elution profiles were monitored by measuring A_{653} (UV-VIS detector SPD-10AV; Shimadzu). Chlorophyll contents were quantified from the chromatographic peak area.

Protein Extraction for Quantitative Analysis

Ten dry seeds of the wild type and the *nyc1/nol* mutant were placed in 1.5-mL tubes containing buffer (125 mM Tris-HCl, pH 8.8, 1% [w/v] SDS, 10% [v/v] glycerol, and 50 mM $Na_2S_2O_5$). The seeds were ground using a glass homogenizer at room temperature until the mixture was homogeneous (usually less than 30 s) and then immediately transferred to ice. Once all the extracts were prepared, the tubes were warmed to room temperature for 5 min to resolubilize the SDS, centrifuged at 13,000*g* for 10 min, and the supernatant was saved (Martínez-García et al., 1999). Quantitative protein analysis was performed using the Bio-Rad Dc Protein Assay.

SDS-PAGE and Immunoblot Analysis

Fifty water-saturated seeds of the wild type and the *nyc1/nol* mutant were homogenized with 200 μL of extraction buffer containing 125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% (w/v) Suc, and 10% (v/v) 2-mercaptoethanol. The homogenates were centrifuged at 10,000*g* for 10 min, and the supernatants (5 μL) were subjected to SDS-PAGE on a 12% (w/v) acrylamide gel. The resolved proteins were subsequently electroblotted onto a Hybond-P membrane (GE Healthcare), and the transferred proteins were detected with anti-LHCII and anti-PsaA/B rabbit primary antibodies and anti-D1 chicken antibody (Agrisera). Anti-rabbit and anti-chicken IgGs linked to horseradish (*Amoracia rusticana*) peroxidase (Santa Cruz Biotechnology) were used as secondary antibodies, and horseradish peroxidase activity was detected using chemiluminescence.

Lipid Extraction

Lipid extraction was based on a modified protocol (Bligh and Dyer, 1959) from the KS Lipidomics Center. Between 10 and 50 mg of seeds was added to 1 mL of 75°C 2-propanol containing 0.01% butylated hydroxyl toluene and heated for 15 min. The samples were transferred to ice for 5 min and then homogenized using a glass homogenizer. The homogenizer was rinsed with 1 mL of chloroform and 1 mL of methanol to remove all the seed parts and lipids. The rinses were combined with the samples and then mixed with 0.8 mL of water. A phase separation was produced by adding 1 mL of chloroform and 1 mL of water. The mixture was centrifuged at 750*g* for 1 min. The chloroform layer was collected, and the aqueous phase was extracted by adding 1 mL of chloroform. The mixture was centrifuged at 750*g* for 1 min. The chloroform and lipid mixture then received 0.5 mL of 1 M KCl. The mixture was centrifuged at 750*g* for 1 min. The chloroform and lipid mixture then received 1 mL of water. The mixture was centrifuged at 750*g* for 1 min. The chloroform and lipid mixture was dried under N_2, and the sample weight was measured.

Thin-Layer Chromatography Analysis

The samples of the lipid extraction were resuspended in 20 times its volume of toluene that contained 0.005% butylated hydroxyl toluene. The lipids were separated on a silica thin-layer chromatography plate (250 μm; Analtech) and developed with hexane:diethylether:acetic acid (60:40:1, v/v; Focks and Benning, 1998).

GUS Analysis

The *NYC1* promoter region was amplified using Arabidopsis genomic DNA by PCR using the primers 5′-CTGCAGCCAACATGCATGTT-3′ (the underlined section is an engineered *Pst*I site) and 5′-GTCGACAGCTATGGAAGAAG-3′ (the underlined section is an engineered *Sal*I site) and cloned into the pGEM-T Easy vector (Promega). The coding region of GUS and the nopaline terminator was amplified using pBI121 (Clontech) by PCR using the primers 5′-GTCGACATGTTACGTCCTGTAGAAAC-3′ (the underlined section is an engineered *Sal*I site) and 5′-CCCGGGCCGATCTAGTAACATAGATG-3′ (the underlined section is an engineered *Sma*I site) and cloned into the pGEM-T Easy vector. The *NYC1* promoter region was inserted into GUS-pGEM between the *Pst*I and *Sal*I sites.

The *NOL* promoter sequence was amplified using the primers 5′-CTGCAGCTCTTTTTAACCCT-3′ (the underlined section is an engineered *Pst*I site) and 5′-GACGTAACATTACAGAAGCTCAAAGAGAGA-3′ (the underlined section anneals *GUS*). The sequence encoding GUS and that for the terminator of nopaline synthase was amplified using the primers 5′-AGCTTCTGTAATGTTACGTCCTGTAGAAAC-3′ (the underlined section anneals the *NOL* promoter) and 5′-CATCTATGTTACTAGATCGGCCCGGG-3′ (the underlined section is an engineered *Sma*I site). The *NOL* promoter-GUS was amplified using these two PCR products by PCR using the primers 5′-CTGCAGCTCTTTTTAACCCT-3′ and 5′-CATCTATGTTACTAGATCGGCCCGGG-3′ and cloned into the pGEM-T Easy vector. The *NYC1* promoter-GUS and *NOL* promoter-GUS were cloned in the pGreenII-0029 vector.

These *NYC1* promoter-GUS and *NOL* promoter-GUS plasmids were subsequently transformed into *Agrobacterium tumefaciens* (strain GV2260) by electroporation using a cuvette with a 1-mm gap at 25 μF and 1.8 kV. Wild-type Arabidopsis was transformed by the floral dip method using standard procedures. The primary transformant Arabidopsis seedlings were selected on agar plates that contained 50 μg mL^{-1} kanamycin as the selection agent.

The GUS activity of the transgenic plants was analyzed with histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-GlcA. The separated organs were incubated with 90% acetone for 15 min and immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcA solution in 100 mM sodium phosphate, pH 7.0, and 0.1% Triton X-100, and the solution was vacuum infiltrated for 30 min. The samples were subsequently incubated at 37°C for 1.5 h. To facilitate the visualization of GUS activity in the plant material, the tissues were cleared by immersing them in 70% ethanol. The leaf and embryo were observed with an S6D microscope (Leica Microsystems).

Electron Microscopy

Electron microscopic evaluation was performed on water-saturated seeds from wild-type and *nyc1/nol* mutant Arabidopsis plants soaked in a primary fixation buffer (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). The seeds were subsequently rinsed three times in 0.1 M cacodylate buffer, pH 7.4, and then fixed with secondary fixation buffer (1% OsO_4 in 0.1 M cacodylate buffer, pH 7.4) for 90 min. To facilitate the removal of water from the tissue, the samples were dehydrated in a graded series of ethanol dilutions (30%, 50%, 70%, 90%, and 100%, three times, v/v) for 15 min at each dilution. After the samples were completely dehydrated, the seeds were embedded in an Epon resin mixture (TAAB Epon 812; TAAB Laboratory Equipment). For electron microscopic observation, ultra-thin sections were stained with 2% (w/v) aqueous uranyl acetate and lead citrate and were subsequently viewed with a 1200EX transmission electron microscope (JEOL).

Seed Germination Assay

As a means to break seed dormancy, wild-type seeds and *nyc1/nol* mutant seeds were sown on saturated filter paper and incubated at 4°C for 3 d. The plates were then placed at 23°C under dark conditions in a growth chamber for 1 d to stimulate seedling germination. The seeds were subsequently observed with an Olympus BX60 microscope to detect germination.

EMSA

The coding region of ABF4 was amplified with an Arabidopsis complementary DNA (cDNA) by PCR using the following primers: 5′-GGATCCTCACCAGTTCCGTATGTGCT-3′ (the underlined section is an engineered *Bam*HI site) and 5′-TTCTTTCAGCTCATTCTTCT-3′. The amplicon was subsequently cloned into the pGEM-T Easy vector and then subcloned into pGEX-3X (GE Healthcare) using the *Bam*HI and *Eco*RI sites. The cDNA was developed by the plant genome project of RIKEN Genomic Sciences Research Complex and was kindly provided by the RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan (Seki et al., 1998, 2002). After confirmation of the sequence integrity, the expression plasmid was then introduced into *Escherichia coli* BL21 cells.

Ten milliliters of an overnight culture of the transformed *E. coli* was diluted with 1 L of Luria-Bertani medium containing ampicillin (50 μg mL^{-1}), and it was grown for an additional 4 h at 37°C. The expression of the recombinant ABF4 protein was induced with the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside for a total induction period of 8 h. After the induction with isopropyl β-D-1-thiogalactopyranoside, the culture was harvested by centrifugation at 10,000*g* for 10 min at 25°C. The collected cells were resuspended in phosphate-buffered saline (100 mM phosphate buffer [pH 7.8] containing 300 mM NaCl) and disrupted by sonication. Sonicated cells were centrifuged at 10,000*g* for 10 min at 4°C, and the supernatant was filtered through a 0.45-μm filter to remove the cell debris. The soluble fraction containing the recombinant ABF4 was loaded onto a GSTrap FF column (GE Healthcare) to enable affinity purification by the glutathione *S*-transferase tag on the ABF4 protein. The unbound proteins received subsequent rinses with phosphate-buffered saline. The affinity-bound recombinant glutathione *S*-transferase::ABF4 fusion proteins were then eluted with 50 mM Tris-HCl (pH 8.0) containing 100 mM reduced glutathione.

For EMSA, the purified recombinant protein (27 μg) was incubated with biotin-labeled double-stranded DNA (100 pmol) fragments (5′-AAACACAAAATGACACGTGTCAATGTTAGGCTAACGAGATGAGGTTGCTGTAACGTGTGG-3′) corresponding to the binding site of ABF4 in the *NYC1* promoter. The binding reactions (20 μL) contained 1× binding buffer (100 mM Tris [pH 7.5], 500 mM KCl, and 10 mM dithiothreitol), 2.5% glycerol, 2.5 mM $MgCl_2$, 50 ng μL^{-1} poly(dI-dC), 0.05% Nonidet P-40, and labeled DNA. The reactions were incubated for 30 min at 25°C, and they were immediately loaded onto a 4% acrylamide gel. The gel was run in 0.5× 45 mM Tris-borate, pH 8.3, 1 mM EDTA at 100 V for 70 min. The resolved DNAs were subsequently electroblotted onto a Hybond-N$^+$ membrane (GE Healthcare) at 380 mA for 30 min, and the membrane was transilluminated under UV for 1,200 × 100 μJ cm^{-2}. The fixed membrane was then blocked with 5% skim milk for 1 h, and detection of the transferred DNA was facilitated by chemiluminescent detection of streptavidin horseradish peroxidase activity.

Fluorescence Spectral Analysis with Confocal Laser Scanning Microscopy

Wild-type seeds and *nyc1/nol* mutant seeds were imbibed in water, and the seeds were subsequently softly broken to detach embryos from the seed coats. The seeds were then analyzed using an Olympus BX60 microscope and a confocal laser scanning microscope (C1si; Nikon) with an excitation

wavelength set at 408 nm. The fluorescence spectra were monitored between 620 and 700 nm.

RNA Isolation and Semiquantitative RT-PCR Analysis

RNA was isolated from Arabidopsis maturing seeds from the pod using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The samples were used to synthesize cDNA using the Prime Script RT Reagent Kit (TaKaRa). PCRs were done with 2 μL of cDNA using KOD FX Neo (Toyobo). Usually, 25 to 29 cycles of PCR were performed, and the annealing temperature was set at 55°C. The primers used for semiquantitative RT-PCR analysis were as follows: for *ACT2*, forward, 5′-ATTGTGCTCAGTGGTGGAAC-3′, and reverse, 5′-CGATTCCTGGACCTGCCTCA-3′; for *Em6*, forward, 5′-TGGCGTCTCAACAAGAGAAG-3′, and reverse, 5′-TCTCCGGTGCTAAGACCA-3′; for *NYC1*, forward, 5′-GCCGATGGTTTGATGACCAA-3′, and reverse, 5′-TCCGAGGAAGATGTTTGTTA-3′.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT4G13250 (NYC1), AT5G04900 (NOL), and AT3G19290 (ABF4).

Supplemental Data

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

Supplemental Figure S1. Phenotype of the *nyc1/nol* mutant seedlings.

Supplemental Figure S2. Protein and lipid contents in wild-type seeds and *nyc1/nol* mutant seeds (fresh and old seeds).

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