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ABI3 controls embryo degreening through Mendel's *I* locus

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Chlorophyll (chl) is essential for light capture and is the starting point that provides the energy for photosynthesis and thus plant growth. Obviously, for this reason, retention of the green chlorophyll pigment is considered a desirable crop trait. However, the presence of chlorophyll in mature seeds can be an undesirable trait that can affect seed maturation, seed oil quality, and meal quality. Occurrence of mature green seeds in oil crops such as canola and soybean due to unfavorable weather conditions during seed maturity is known to cause severe losses in revenue. One recently identified candidate that controls the chlorophyll degradation machinery is the stay-green gene, *SGR1* that was mapped to Mendel's *I* locus responsible for cotyledon color (yellow versus green) in peas. A defect in *SGR1* leads to leaf stay-green phenotypes in *Arabidopsis* and rice, but the role of *SGR1* in seed degreening and the signaling machinery that converges on *SGR1* have remained elusive. To decipher the gene regulatory network that controls degreening in *Arabidopsis*, we have used an embryo stay-green mutant to demonstrate that embryo degreening is achieved by the *SGR* family and that this whole process is regulated by the phytohormone abscisic acid (ABA) through ABSCISIC ACID INSENSITIVE 3 (ABI3); a B3 domain transcription factor that has a highly conserved and essential role in seed maturation, conferring desiccation tolerance. Misexpression of *ABI3* was sufficient to rescue cold-induced green seed phenotype in *Arabidopsis*. This finding reveals a mechanistic role for ABI3 during seed degreening and thus targeting of this pathway could provide a solution to the green seed problem in various oil-seed crops.

freezing tolerance | nondormant

The success of angiosperms impinges on their ability to desiccate and protect their embryos in a dormant state until favorable conditions are perceived. In many angiosperms and oil-seed plants such as *Arabidopsis* and canola, this desiccation process during seed maturation is intricately coupled to loss of chlorophyll (chl) from photosynthetically active embryos (1). During the embryo maturation phase, as the embryos begin to lose their chlorophyll, they concomitantly initiate the process of acquisition of desiccation tolerance and dormancy, thereby producing mature, brown (degreened) and dormant seeds. The persistence of chlorophyll in mature seeds has negative impacts on seed storability in many commercial plant species such as canola, cabbage, carrot, geranium, and soybean (2–4). Apart from contributing to reduced storability, prevalence of green seeds in mature oil seeds (canola and soybean) is also associated with reduced shelf life of oil and production of unfavorable odors and flavors. Particularly, in canola, which is one of the major global cash crops, the frost-induced green seed problem has been estimated to result in an annual loss of $150 million in revenue in North America alone (5, 6).

During seed development, abscisic acid (ABA) is known to control mid to late stages of embryo maturation and desiccation tolerance through the B3 domain transcription factor ABSCISIC ACID INSENSITIVE 3 (ABI3) (7). The severe *ABI3* allele, *abi3-6* exhibits pleiotropic effects during seed development that bypass the embryo maturation phase and transition directly into the germination and vegetative phases (8). This lack of embryo maturation phase results in seeds that remain green due to failure of embryo degreening in the severe *abi3* alleles. These green seeds are nondormant, desiccation intolerant, and display extreme ABA insensitivity. ABA signaling through SUCROSE NONFERMENTING 1 (SNF1)-related protein kinases (SnRKs) is also required for this degreening process as triple *SnRK* mutants (*snrk* d/e/i) harbored mature green seeds that were viviparous and desiccation intolerant, similar to *abi3-6* seeds (9).

In peas, Mendel's *I* locus, which codes for *SGR* (stay green) gene was shown to be responsible for cotyledon color (yellow versus green) (10, 11). Interestingly, green pea seeds defective in *SGR1* have not been reported to have any longevity problems compared with their yellow counterpart. Stay-green mutants resulting from defects in *SGR* belong to the type C class of nonfunctional stay-green mutants that proceed normally with the loss of photosynthesis and senescence process despite the presence of high levels of chlorophyll (12). In *Arabidopsis*, there are two *SGR* genes, *SGR1* (At4g22920) and *SGR2* (At4g11910). Loss-of-function null mutations in the *SGR1* did not result in green embryos, although a stay-green vegetative leaf phenotype was reported (13, 14). It still remains unexplored whether the *Arabidopsis SGR* orthologs participate in the seed degreening process similar to Mendel's *I* locus.

The lack of seed degreening in *abi3-6* mutants suggests that ABI3 should function as a master regulator of seed degreening. Thus, this genotype is an excellent starting material to understanding embryo degreening. By analyzing the transcriptional landscape of maturing *abi3-6* seeds, we identified that the *SGR*

Significance

Occurrence of mature green seeds in oil-seed crops such as canola and soybean causes severe losses in revenue. Retention of chlorophyll in seeds can be an undesirable trait as it affects seed maturation, seed oil, and meal quality. We show that the abscisic acid (ABA, plant hormone) dependent transcription factor ABSCISIC ACID INSENSITIVE 3 (ABI3), confers seed degreening by regulating Mendel's stay-green genes. This study unveils a new role for ABI3 in removal of seed chlorophyll in addition to its functions in embryo maturation and conferring desiccation tolerance. This pathway could be manipulated to tackle the cold-induced green seed problem in oil-seed crops.

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gene family is under direct transcriptional control of *ABI3*. Moreover, we show that *SGR2*, but not *SGR1*, mediated embryo-specific degreening. Misexpression of *SGR* genes fully rescues the stay-green phenotype and partially rescues the ABA insensitivity of *abi3-6* but does not rescue other late embryogenesis defects such as dessication intolerance and seed storage protein accumulation in this allele. Thus, the pleiotropic phenotypes associated with *ABI3* mutants can be separated into two programs, one involving defects in degreening and the other involving aberrant seed maturation.

Results

***abi3-6* Is an Embryo Stay-Green Mutant.** Although multiple *ABI3* alleles have been identified with various degrees of ABA insensitivity, not all of these alleles display the green seed phenotype exhibited by the severe alleles of *ABI3*. To precisely examine the various stages of degreening during embryo maturation phase, we tagged flowers from Col-0, *abi3-8* (weak *abi3* allele), and *abi3-6* right after fertilization, followed by harvesting and comparison of the embryos at various stages postfertilization indicated by days after flowering/fertilization (DAF). Both Col-0 and *abi3-8* displayed complete embryo degreening at 16 DAF, whereas *abi3-6* embryos still remained green, confirming the previously documented stay-green embryo phenotype observed only with the severe alleles of *ABI3* (Fig. 1*A*).

abi3-6 was originally isolated from a fast neutron screen on inhibiting concentration of ABA (8). Sequencing of the *ABI3* transcripts from *abi3-6* revealed a premature stop codon caused by the deletion, leading to abrupt stoppage of the ORF after amino acid 231 (*SI Appendix*, Fig. S1*A*). Thus, *ABI3-6* codes for a truncated, short protein with only the intact A1 domain without the B1, B2, and B3 domains (*SI Appendix*, Fig. S1*A*). The severe *abi3-6* phenotype could result from lack of the DNA binding B3 domain due to the truncation and the inability of ABI3-6 to localize to the nucleus as observed from transient expression in tobacco cells (15) (*SI Appendix*, Fig. S1*B*). This is in contrast to the weaker, leaky phenotype and nuclear localization displayed by point mutations in *ABI3* (16) (*SI Appendix*, Fig. S1 *A* and *B*).

Transcriptional Landscape of *abi3-6* Embryos Reveals Repression of Mendel's *I* Locus. In *Arabidopsis*, ABI3 is highly expressed in the embryo throughout the maturation phase (17) (*SI Appendix*, Fig. S1*C*). We hypothesized that if lack of ABI3 function results in the embryo stay-green phenotype, then the expression of downstream ABI3 targets will be affected and the factors responsible for embryo-degreening should represent a subset of the targets regulated by ABA. To examine this, we performed microarray analyses to identify the transcriptome profile of embryos in their late maturation phase (13 DAF) when embryos begin to enter the degreening phase (13–16 DAF) and when ABI3 expression is maximal (*SI Appendix*, Fig. S1*C*). Microarray was performed with RNA extracted from embryos harvested from siliques at 13 DAF. From the microarray analyses (http://mapman.gabipd.org/web/guest/robin) (18), we identified several groups of genes that were expressed at least twofold higher or lower in *abi3-6* mutant than in

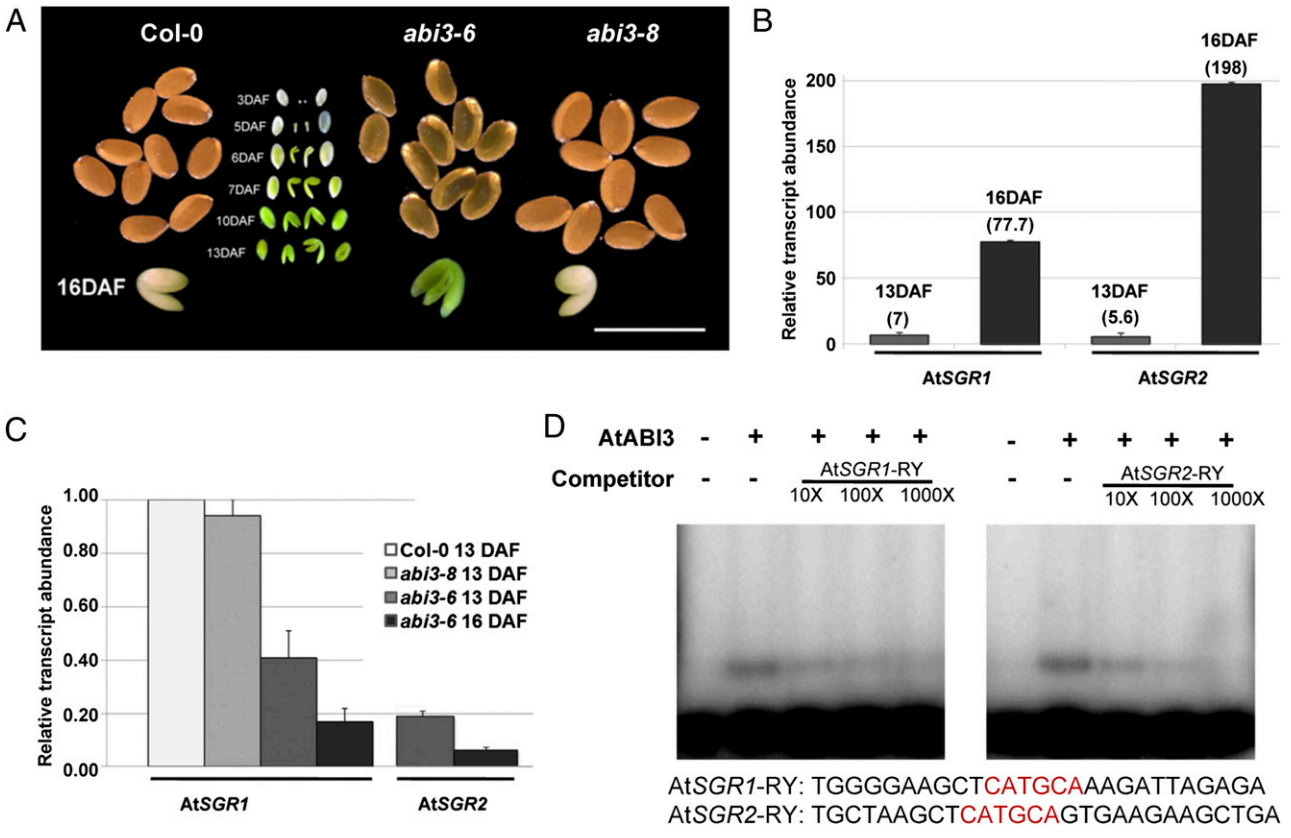

Fig. 1. *SGR1* and *SGR2* are up-regulated during seed maturation and are reduced in *abi3-6* background. (*A*) Seed and mature embryo phenotypes of *abi3-6* and *abi3-8* mutants compared with Col-0 (Scale bar, 1 mm.) (*B*) Up-regulation of *SGR1* and *SGR2* expression in Col-0 embryos between 13 and 16 DAF. Values in parentheses indicate expression relative to At*TUB4* expression. (*C*) *SGR1* and *SGR2* expression in *abi3-6* and *abi3-8* mutants relative to Col-0, normalized against At*TUB4*. Values are means ± SE of three biological replicates. (*D*) Electrophoretic mobility shift assays (EMSAs) of recombinant ABI3 B3 domain protein with ^{32}P-labeled probes derived from the *SGR1* and *SGR2* gene promoters. Competition experiments were performed using increasing amounts (10×, 100×, 1000×) of the *SGR1*/*SGR2* unlabeled probes. The B3-binding target sequence is shown below the autoradiographs.

Col-0 (*SI Appendix*, Fig. S2). Seed storage proteins and late embryogenesis abundant proteins were repressed, whereas genes related to light reactions and tetrapyrrole synthesis, which are involved in photosystem organization and chlorophyll biosynthesis, were induced in *abi3-6* (*SI Appendix*, Table S1). Interestingly, Mendelian stay-green locus At*NYE1*/*SGR1* was expressed at a threefold lower level compared with Col-0 ($\log_2$ fold change −1.54) (*SI Appendix*, Table S1).

SGR1 and _SGR2_ Are Up-Regulated During Seed Maturation and Reduced in _abi3-6_ Background. Defects in *SGR1* lead to stay-green leaf phenotypes in *Arabidopsis* and rice and a stay-green cotyledon phenotype in peas (10). Currently, there is no evidence to suggest that *SGR1* is also responsible for embryo degreening in *Arabidopsis* and other related species. In public microarray databases, At*SGR1* (the only *SGR* family member represented in the microarray database) expression in maturing seeds mirrored *ABI3* expression and was up-regulated following increase in *ABI3* expression (*SI Appendix*, Fig. S1*C*). Reverse transcription-quantitative PCR (RT-qPCR) was performed to validate the microarray results and to investigate the changes in expression patterns of both *SGR1* and *SGR2* during seed maturation. During seed maturation in Col-0, expression of both *SGR1* and *SGR2* are highly up-regulated between 13 and 16 DAF, with *SGR1* and *SGR2* displaying a 10- and 35-fold increase, respectively (Fig. 1*B*). On 13 DAF, compared with Col-0, both *SGR1* and *SGR2* had lower expression in *abi3-6* (Fig. 1*C*). In *abi3-6* seeds, *SGR1* levels are 2.4-fold and 6-fold lower than Col-0 at 13 and 16 DAF, whereas *SGR2* levels are lower by 5-fold and 17-fold at 13 and 16 DAF, respectively (Fig. 1*C*). While these results validate the microarray findings, they also indicate a lack of up-regulation of *SGR* family members in the absence of functional ABI3.

B3 DNA Binding Domain of ABI3 Interacts with the RY _Cis_-Motif of _SGR1_ and _SGR2_ Promoter Sequences. To determine if the down-regulation of At*SGR1/2* in the *abi3-6* embryo is a direct consequence of lack of ABI3-mediated transcriptional activation of *SGR1/2*, we analyzed the promoter sequences of both *SGR1* and *SGR2* genes. Both promoters possessed the canonical B3 domain binding RY motif CATGCA with variable flanking nucleotides. Electrophoretic mobility shift assays (EMSAs) were performed using purified recombinant ABI3 B3 domain with labeled RY sequence motif (27 nt) of *SGR1* and *SGR2*. Coincubation of ABI3 B3 domain and labeled RY sequences resulted in retardation of the protein–nucleotide complex, indicating binding of ABI3 B3 domain with RY sequences of *SGR1* and *SGR2* (Fig. 1*D*). The binding specificity was further verified through addition of unlabeled RY sequences (10, 100, and 1000×) in the reaction that resulted in reduction to complete elimination of signal from the autoradiograph (Fig. 1*D*).

Ectopic Expression of Either _SGR1 or SGR2_ Rescues _abi3-6_ Degreening Defect. If reduced levels of *SGR1/2* transcripts were responsible for the observed stay-green embryo phenotype of *abi3-6* mutant, then misexpression of *SGR* in *abi3-6* background would restore the embryo degreening process. To test this hypothesis, we ectopically expressed *SGR1* and *SGR2* under the control of cauliflower mosaic virus (CaMV) 35S promoter in *abi3-6* background. When T1 transgenic lines were examined, both overexpressions (*abi3-6/35S::SGR1*, *abi3-6/35S::SGR2*) resulted in restoration of embryo degreening (Fig. 2 *A* and *B*), although overexpression of *SGR1* caused pleiotropic shoot phenotypes with typical yellowing of leaves (*SI Appendix*, Fig. S3*A*), as previously reported (13). In contrast, *SGR2* misexpression conferred embryo-specific degreening phenotype in *abi3-6* seeds without any shoot yellowing phenotype (Fig. 2*B* and *SI Appendix*, Fig. S3*B*). This indicates a seed-specific role for *SGR2* during embryo degreening.

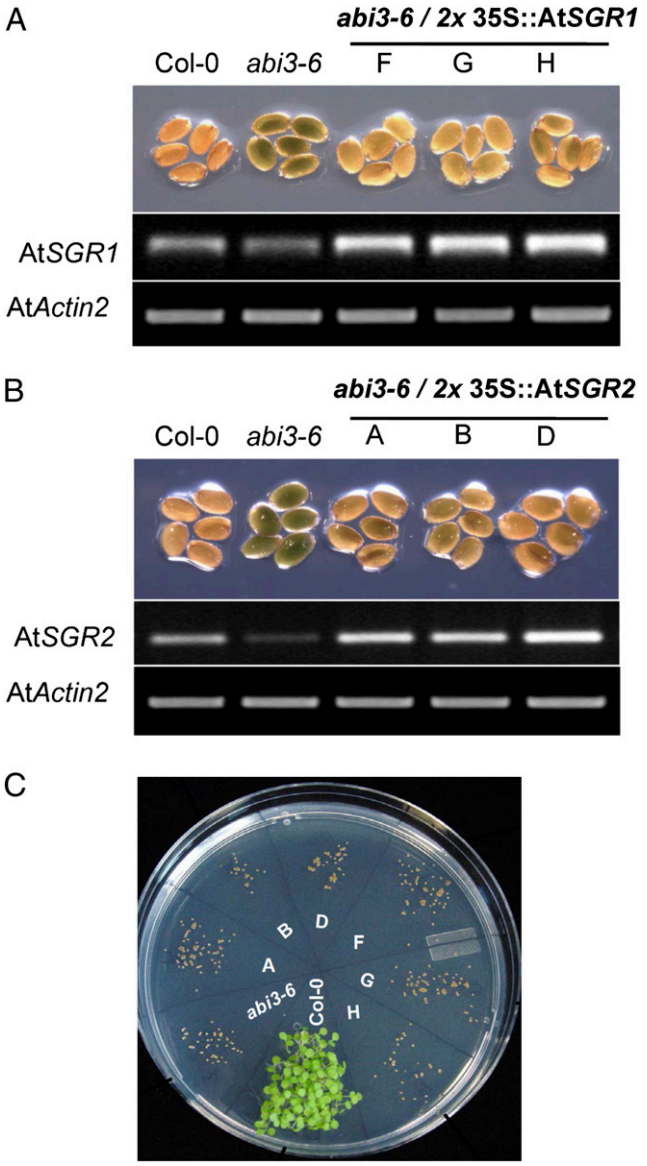

Fig. 2. *SGR1* and *SGR2* overexpression rescues *abi3-6* stay-green embryo phenotype. Phenotype of three representative lines (F–H) from *abi3-6*/35S::*SGR1* (*A*) and *abi3-6*/35S::*SGR2* (A, B, and D) (*B*) showing rescue of embryo degreening. The respective RT-PCR analysis to verify *SGR1 and SGR2* overexpression is shown at bottom of *A* and *B*. (*C*) Seed desiccation tolerance assay. Mature seeds from Col-0, *abi3-6*, and the *abi3-6/35S::SGR* overexpressors (A, B, D, and F–H) were stored for 4 wk followed by stratification and germination on 0.5× MS plates to assess desiccation tolerance.

SGR-Mediated Degreening Is Partially Coupled to ABA Insensitivity but Not Desiccation Tolerance. Three independent *abi3-6/35S::SGR1*, *abi3-6/35S::SGR2* overexpressors were further examined to test if any of the pleiotropic phenotypes associated with the *abi3-6* lesion are altered following overexpression of *SGR1* or *SGR2*. When mature brown seeds from these *abi3-6/35S::SGR* overexpressors were collected and stored for 4 wk, they were completely incapable of germination even after prolonged stratification (Fig. 2*C*), mimicking the *abi3-6* phenotype. This indicates that the lack of desiccation tolerance in *abi3-6* occurs independent of the degreening process. When protein levels in these seeds were examined, the various seed storage protein levels in *abi3-6/35S::SGR* overexpressors resembled the *abi3-6* profile, revealing that the

accumulation of the storage proteins was still defective in these brown seeds (*SI Appendix*, Fig. S4*A*).

To assess if the degreening defect of *abi3-6* was associated with the observed extreme ABA sensitivity, the brown, viable, predesiccation seeds from the *abi3-6*/*35S::SGR* overexpressors were assessed for their ability to germinate at either 10 or 25 μM of ABA. At 24-h postplating, the transgenic lines exhibited relatively increased sensitivity to both concentrations of ABA compared with *abi3-6*, but at 72-h postplating, almost all of the seeds had germinated at both concentrations of ABA (*SI Appendix*, Fig. S4 *B* and *C*). Thus, degreening seems to marginally influence ABA sensitivity of seeds.

SGR1 and SGR2 Are Necessary for Seed Degreening in *Arabidopsis*. Rescue of the *abi3-6* degreening defect through *SGR1/2* overexpression shows that *SGR1* and *SGR2* are sufficient to drive degreening in maturing *abi3-6* embryos. To prove necessity of *SGR1* and *SGR2* in embryo degreening, we analyzed single and double mutants of *SGR1* and *SGR2*. RT-PCR analysis of seeds at 16 DAF revealed that in *sgr1-1* there was reduced expression of *SGR1*, whereas *sgr2-2* plants had a complete absence of *SGR2* transcripts (Fig. 3*A*). The double mutants (*sgr1-1*/*sgr2-2*) mimicked the single mutants in *SGR1* and *SGR2* expression. Interestingly, in the single mutants, we observed an increase of *SGR2* in *sgr1-1* background and vice versa, suggesting a compensatory increase in the transcript of the other *SGR* member in the single mutants (Fig. 3*A*). When observed for seed degreening defects at various time points postfertilization, we observed no difference in seed degreening in the single mutants compared with Col-0 (Fig. 3*B*). However, the double mutants clearly displayed persistence of chlorophyll at stages when Col-0 and the single mutants were progressing through the degreening process (Fig. 3*B*). This green seed phenotype was maintained at maturity and also in stored seeds (Fig. 3*C*). When seeds stored for 4 wk were assayed for chlorophyll content, the double mutants had significantly high levels of chlorophyll (Fig. 3*C*). Interestingly, in contrast to *abi3-6* green seeds that were incapable of germinating after 4 wk of storage, the *sgr1-1*/*sgr2-2* seeds (after 4 wk of storage) showed 100% germination following stratification (Fig. 3*D*). When assayed for ABA sensitivity, *sgr2-2* and *sgr1-1*/*sgr2-2* had significantly higher radicle emergence at 2 μM ABA compared with *sgr1-1* and Col-0 (Fig. 3*D*). The ability of green *sgr1-1*/*sgr2-2* seeds to germinate prompted us to investigate if the seed storage protein levels are altered in the green seeds. Protein gels revealed that lack of *SGR1* and *SGR2* did not affect the ability of the seeds to accumulate the storage proteins as the single and double mutants mimicked the Col-0 protein expression pattern, whereas *abi3-6* seeds showed a clear lack of accumulation of these proteins (*SI Appendix*, Fig. S5). Thus, *SGR1* and *SGR2* are required for embryo degreening during seed maturation and this degreening process is independent of seed storage protein accumulation, although degreening seems to promote ABA sensitivity of mature brown seeds.

ABI3 Overexpression Rescues Freezing-Induced Green Seeds in *Arabidopsis*. Frost is one of the major factors that can cause fixation of green color in mature seeds. Exposure of maturing canola plants to sublethal frost of 0 °C to 1 °C can fix the green color in seeds, resulting in major losses in the canola industry (3, 4). Based on our identification of ABI3 as the master regulator of degreening, we hypothesized that if freezing resulted in the down-regulation of chlorophyll degradation pathway, overexpression of *ABI3* under a constitutive promoter should allow circumventing of this problem.

To test this, we investigated whether *Arabidopsis* can mimic the frost-induced green seed phenotype observed in canola. Exposure of maturing *Arabidopsis* pods at various days after flowering (11–13 DAF) to freezing temperatures resulted in mature green seeds (Fig. 4*A*). When trangenic *Arabidopsis* lines overexpressing *ABI3* in *abi3-6* background (*35S::ABI3*/*abi3-6*) were subjected to a similar treatment, the transgenic seeds proceeded to degreen to produce mature brown seeds despite the cold treatment (Fig. 4*A*). Our RT-qPCR analyses revealed that

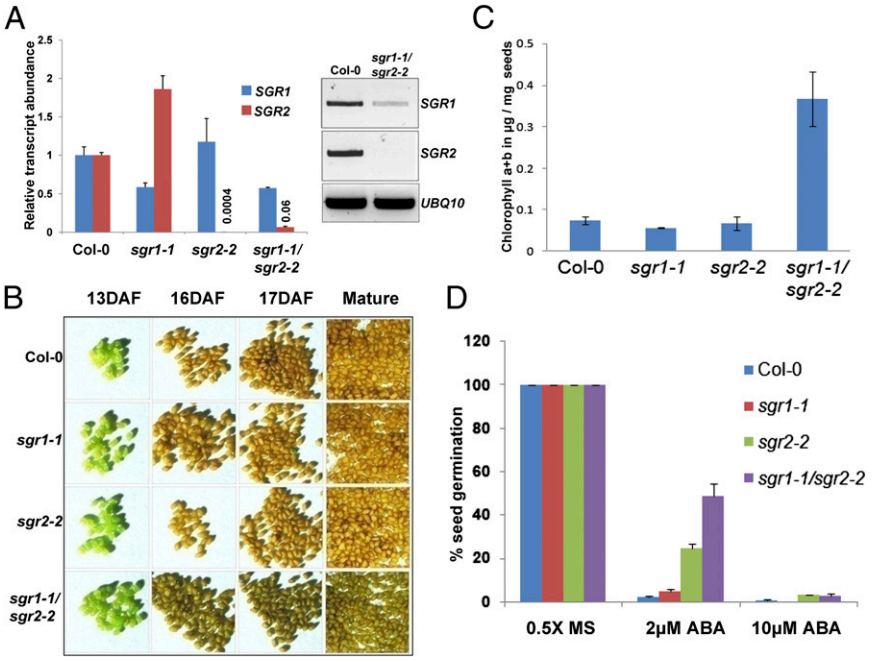

Fig. 3. SGR1 and SGR2 play a redundant function in embryo degreening. Col-0, *sgr1-1*, *sgr2-2*, and *sgr1-1/sgr2-2* mutants were analyzed for (*A*) *SGR1* and *SGR2* expression at 16 DAF through either RT-qPCR (*Left*) or RT-PCR (*Right*). Values indicate levels of *SGR2* transcripts relative to Col-0. (*B*) Seed degreening from 13 DAF to 17 DAF and at maturity (desiccated) in planta. (*C*) Chlorophyll accumulation in mature seeds. (*D*) ABA sensitivity (2 μM and 10 μM ABA) following storage for 4 wk. Values are means ± SEM of three biological replicates.

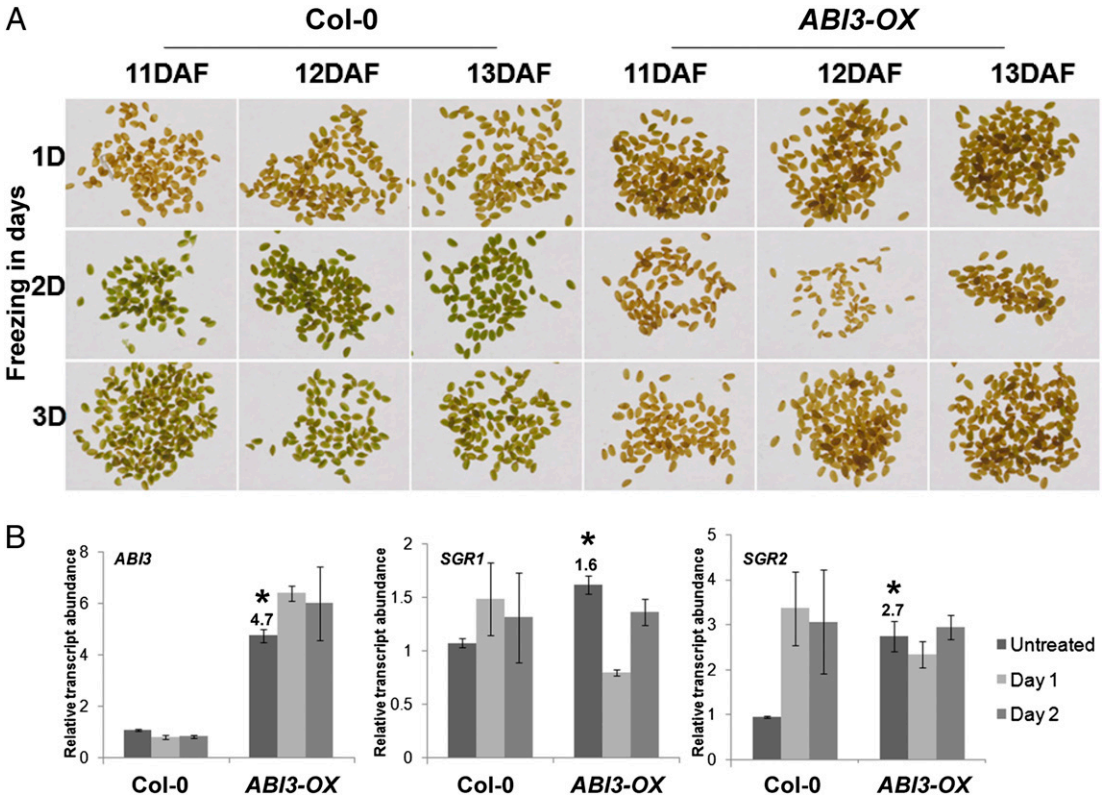

Fig. 4. ABI3 overexpression rescues cold-induced green seeds in *Arabidopsis*. (*A*) Mature seeds from Col-0 and ABI3 overexpressing (*35S::ABI3/abi3-6*) plants showing cold-induced effect on degreening. *Arabidopsis* plants were exposed to −5 °C to −10 °C (2 h/d) for 1–3 d at various stages of pod development, followed by maintenance at ambient temperature (22 °C). Following maturation, the seeds were harvested and observed for presence of green seeds. (*B*) RT-qPCR analysis of *ABI3* (*Left*), *SGR1* (*Center*), and *SGR2* (*Right*) expression in seeds (11–13 DAF) either left untreated or exposed to freezing as in *A* and allowed to recover for either 1 d or 2 d at ambient temperature ($n = 4$). *$P < 0.05$ compared with untreated Col-0. Values indicate abundance of respective transcripts relative to untreated Col-0.

the ABI3 overexpressing lines constitutively expressed significantly higher levels of *ABI3*, *SGR1*, and *SGR2* compared with Col-0 before exposure to freezing (Fig. 4*B*). *SGR2* was induced in maturing seeds when Col-0 plants were exposed to freezing temperatures and allowed to recover for 1 or 2 d, whereas similar treatment only had a mild effect on expression of *ABI3*, *SGR1*, and *SGR2* in ABI3-OX lines (Fig. 4*B*).

Discussion

Through using the green seed mutant *abi3-6*, we have identified the genetic regulatory network that controls seed degreening in *Arabidopsis*. We have demonstrated that during embryo development, ABI3 functions in two independent programs; one that regulates seed degreening through the *SGR* family and the other that is dedicated to seed maturation and desiccation tolerance (Fig. 5). Furthermore, our study demonstrates that the influence of ABI3 on chlorophyll degradation is seed specific, because *abi3-6* plants did not display a stay-green leaf phenotype similar to *sgr1-1* when kept under dark conditions (*SI Appendix*, Fig. S6). Thus, the transcriptional activation of *SGR1/2* by ABI3 forms an exclusive seed-specific degreening module that is required for successful embryo degreening.

Interestingly, retention of chlorophyll in seeds has been considered to be a detriment to the embryo due to the phototoxic nature of chlorophyll and its catabolites. The *sgr1-1/sgr2-2* double mutants, however, exhibited normal desiccation tolerance, were able to acquire dormancy, and germinated 100% after storage (Fig. 3). During senescence or leaf yellowing, loss of chlorophyll is initiated when chl a and chl b are broken down through the action of pheophytinase and pheide a oxygenase (PaO) into red chlorophyll catabolites (19). Lack of PaO is known to result in light-dependent accelerated cell death phenotypes due to accumulation of catabolites in the chl-degradation pathway that are phototoxic (20–23). In the leaf, *SGR1* functions upstream of *PaO* as *sgr1* mutants do not accumulate any phototoxic catabolites that accumulate in *pao* mutants (14). This model of *SGR1* functioning

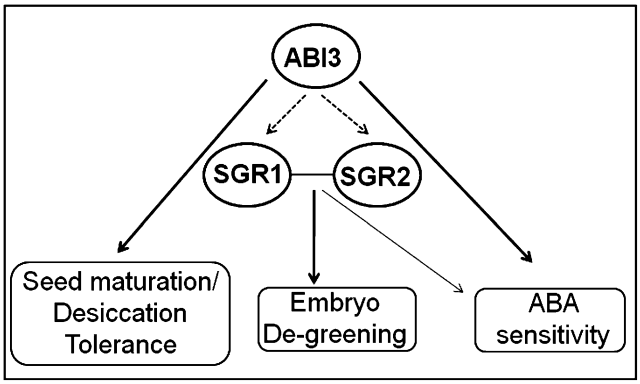

Fig. 5. Genetic network regulating embryo degreening in *Arabidopsis*. Embryo degreening is exclusively orchestrated by ABI3. ABI3 controls embryo degreening through regulating transcription of functionally redundant *SGR1* (Mendel's *I* locus) and *SGR2*, which function downstream of ABI3 to mediate degreening. This degreening process is also partially coupled to ABA sensitivity. Dashed arrows represent transcriptional regulation. Thin arrows represent partial or lesser control.

upstream of *PaO* would explain the viability of mature *sgr1-1/sgr2-2* green seed phenotype. By contrast, ABA sensitivity in both *abi3-6/SGR* overexpressors and *sgr1-1/sgr2-2* double mutants suggested the presence of chl could influence sensitivity of seeds to ABA. *abi3-6/35S::SGR* overexpressors, other than rescuing the green seed phenotype, also reduced the ABA insensitivity of *abi3-6* (Fig. 2). In contrast, in *sgr1-1/sgr2-2* mutants, lack of *SGR1* and *SGR2* resulted in marginal ABA insensitivity (Fig. 3). The *grs* enhancer mutation that results in green seeds in the weak *abi3-1* background did not alter ABA insensitivity of *abi3-1*, but these seeds were affected in their longevity (2) unlike the *sgr1-1/sgr2-2* mutants. Whether the *grs/abi3-1* mutant accumulates phototoxic chl catabolites that affect seed longevity or the mechanism behind how *grs* mutation enhances *abi3-1* phenotype are not known. The chloroplast localized, ABA binding, Mg-chelatase H subunit, CHLH, has been shown to participate in ABA responses independent of chlorophyll biosynthesis (24). Overexpression of CHLH resulted in increased sensitivity to ABA, without altering chlorophyll levels (24), whereas the chl-deficient *chlh* (*cch*) mutant was insensitive to ABA (24). Thus, proteins involved in chl biosynthesis (CHLH) or degradation (SGR) could also influence ABA responses, although the insensitivity displayed is quite weak compared with strong ABA insensitive mutants such as *abi3-6*.

Misexpression of ABI3 was sufficient to rescue the cold-induced green seeds in *Arabidopsis* (Fig. 4). There is precedence for overexpression of ABI3 conferring freezing tolerance to plants (25). These studies, which only focused on vegetative tissues, found overexpression of ABI3 resulted in increased ABA sensitivity of *Arabidopsis* leaves and accumulation of ABA-dependent, ABI3-regulated transcripts such as *RAB18* (25–27). In our study, we found ABI3 overexpressors, accumulated *RAB18* as well as increased levels of *SGR1* and *SGR2* (*SI Appendix*, Fig. S7). One possibility is that the misexpression of ABI3 results in increased sensitivity of these lines to endogenous ABA, which in turn leads to priming for freezing and desiccation tolerance within the seed. Alternatively, the increased expression of ABI3 may directly alter ABI3-dependent transcripts required for chlorophyll degradation (*SGR1* and *SGR2)* and freezing/desiccation tolerance (*RAB18*) (Fig. 4*B* and *SI Appendix*, Fig. S7). This ABI3-dependent priming allows these seeds to withstand the freezing treatment and continue to degreen to produce mature brown seeds. Of course, these two models are not mutually exclusive and both ABA sensitivity and ABI3 may be required for the seed priming and freezing tolerance. Whatever the case, in either an ABA or ABI3 centric model, the lack of basal accumulation of these transcripts in wild-type seeds results in a freezing-sensitive system that is defective in the chlorophyll degradation process following cold treatment. Given that green seed problem is a major industry concern in oil-seed crops such as canola (3, 4), our identification of ABI3 as the master regulator of this pathway should allow development of strategies to exploit this pathway to tackle the green seed problem. Isolation of the canola *ABI3* ortholog and overexpression of Bn*ABI3* through either a constitutive promoter or a seed-specific cold-inducible promoter could provide a viable solution to the green seed problem associated with oil seeds.

Nevertheless, through using the *abi3-6* embryo stay-green mutant, we have identified that during embryo maturation, ABI3 functions as the master regulator of degreening through transcriptional control of *SGR1* and *SGR2* (Fig. 5). This ABI3-mediated degreening process is independent of acquisition of desiccation tolerance and is partially coupled to ABA sensitivity.

Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis thaliana* ecotype Col-0 was used as the wild type in this study. *abi3-6* and *abi3-8* mutant lines were isolated from fast neutron- and EMS-mutagenized M2 populations of ecotype Columbia, respectively (16). Single transfer DNA (T-DNA) insertional mutants of *SGR1* (At4G22920, SALK_070891, *sgr1-1*) and *SGR2* (AT4G11910, SALK_003830, *sgr2-2*) were obtained from the *Arabidopsis* Biological Resource Center and genotyped through PCR to isolate homozygous lines. These lines were crossed and F2 generation was genotyped to isolate the *sgr1-1/sgr2-2* double mutants. Plant media and growth conditions were done as previously described (28). Siliques were staged for phenotypic analysis of whole embryos and for microarray analysis by tying colored thread around the pedicel on the day of flower opening. Intact siliques were harvested on the indicated number of DAF and seeds were observed for degreening or excised with a needle and used for RNA extraction and microarray analysis.

Plant Transformation for Complementation of the *abi3-6* Mutant. *A. thaliana* At*SGR1*, At4g22920, AY850161, and At*SGR2*, At4g11910, AY699948 were cloned into the pGEM-T vector (Promega). The excised DNA fragments were cloned into the T-DNA binary vector pCAMter (2X35S::cDNA). The various constructs carrying *SGR1* or *SGR2* cDNAs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation and used for floral dip transformation (29) of *abi3-6* mutant plants. Harvested seeds were spread on MS medium containing kanamycin for selection of transgenic plants.

Transient Expression Using BY-2 Cells. Biolistic bombardments of cultured tobacco (*Nicotiana tabacum*) BY-2 cells were performed essentially as described previously (30). The full-length *ABI3* cDNA (At3g24650), *ABI3-6*, and *ABI3-8* cDNAs were cloned into the expression vector pRTL2 under the control of a CaMV 35S promoter for expression as GFP-tagged proteins. These constructs were bombarded into BY-2 cells. Cells were fixed with 4% (wt/vol) paraformaldehyde and visualized directly through fluorescent microscopy for detecting GFP.

Microarray Analysis. Seeds from wild-type Col-0 and *abi3*-6 were excised from siliques at 13 DAF. Total RNA was extracted and triplicate microarray analysis using independent seed batches was performed. For each sample analyzed, 5 μg of total RNA was converted to biotin-labeled cRNA using oligo(dT) priming as described by the manufacturer (Enzo kit; Affymetrix) and hybridized to 22K ATH1 Affymetrix microarrays at the Affymetrix Genechip facility (University of Toronto). Microarray data were statistically analyzed with the flexible user friendly graphical interface ROBIN (http://mapman.gabipd.org/web/guest/robin) to generate log-fold change of differential gene expression. Fold changes of significantly differentially expressed genes ($P < 0.01$) were analyzed with the pathway analysis program MapMan (18) (https://gabi.rzpd.de/projects/MapMan/) to map large datasets into diagrams and processes. A color code is used to symbolize the fold change of differential gene expression, where blue indicates higher expression, red indicates lower expression, and white indicates no change in *abi3-6* mutant embryos (*SI Appendix*, Fig. S2).

Reverse Transcription Quantitative PCR. Total RNA from seeds was prepared using RNAqueous columns with the Plant RNA isolation aid (Ambion) according to manufacturer instructions. Total RNA samples were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies). RNA was additionally analyzed by gel electrophoresis to confirm integrity. RT reactions were performed using SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's directions with 2 μg total RNA input. Quantitative PCRs (qPCRs) were performed using a Chromo 4 real-time PCR detector (Bio-Rad) controlled by Opticon Monitor 3 software. Standard curves were generated using five concentrations in triplicate in a dilution series, followed by the test sample run with three biological samples (each analyzed with triplicate technical replicates). qPCR results were quantified by the Pfaffl method as described in the real-time PCR applications guide (Bio-Rad). The Col-0 samples were used as calibrators. The target genes were normalized against the reference genes β-tubulin (*TUB4*) or ubiquitin 10 (*UBQ10*). Stability of four reference genes (*TUB4*, *UBQ10*, *ACT2*, and *GAPDA*) was analyzed under the various conditions used in this study using the BestKeeper program (31) to identify the most stable reference genes. In developing seeds (13 DAF and 16 DAF), *TUB4* expression was highly stable in both Col-0 and *abi3-6* genotypes (*SI Appendix*, Table S2 and Fig. S8). *UBQ10* was used as the reference gene for cold-treated samples because it was the most stable reference gene following cold treatments (*SI Appendix*, Table S3 and Fig. S9). Primers used in this study are listed in *SI Appendix*, Table S4.

Electrophoretic Mobility Shift Assays. Mobility shift assays were performed as described previously (32, 33). The sequences of all of the oligonucleotides are shown in Fig. 1*D*. Both strands of the oligonucleotides were synthesized and annealed. DNA probes were generated by filling in 5′ overhangs with the

Klenow fragment of DNA polymerase I (Promega) in the presence of [α-^{32}P] dATP and purified using MicroSpin columns (GE Healthcare). Double-stranded DNA with nonradioactive nucleotides were used as competitor DNA. For the gel shift assays either ~200 pmol of the labeled probe or various concentrations of unlabeled probe along with the labeled probe were incubated with 50 ng of purified recombinant ABI3-B3 DNA binding domain in 2× reaction buffer [12 mM Hepes, 1 mM $MgCl_2$, 4 mM Tris, pH 7.9, 100 mM KCl, 0.6 mM DTT, and 12% glycerol (vol/vol)]. The samples were incubated for 30 min at room temperature and separated by 5% nondenaturing gel (0.5× TBE buffer) at 4 °C. Following electrophoresis, the gel was dried and subjected to autoradiography.

ABA Sensitivity Assays. ABA was dissolved in ethanol and added to the media after sterilization. Mature seeds from Col-0, *abi 3–6*, and *abi3-6/SGR* overexpressors were harvested and tested for ABA sensitivity (germination scored as radicle emergence) without stratification. Seeds were tested for germination on half strength Murashige and Skoog (MS) media (without sucrose supplement), in the presence of 10 μM or 25 μM ABA at room temperature. The double *sgr1-1/sgr2-2* mutant seeds were analyzed for ABA sensitivity along with the respective single mutants and Col-0 on half strength MS media (without sucrose supplement) with 2 μM and 10 μM ABA. Values are means ± SEM of three biological replicates.

Seed Protein Analysis. Seed protein was extracted by grinding mature seeds in an ice-cold mortar with 20 $\mu L \cdot mg^{-1}$ seed of extraction buffer [100 mM Tris·HCl, pH 8.0, 0.5% SDS, 10% glycerol (vol/vol) and 2% (vol/vol) β-mercaptoethanol]. Extracts were boiled for 3 min and centrifuged. Equal amounts of proteins were resolved by SDS/PAGE using a 10% gel. Proteins were visualized by Coomassie blue staining.

Cold-Induced Degreening Assays with *Arabidopsis*. Flowers from *Arabidopsis* plants (Col-0 and *35S::ABI3/abi3-6*) were tagged on the day of flower opening to indicate the day of fertilization/flowering. At various days after flowering (11, 12, and 13 DAF), these plants were subjected to freezing (−5 °C to −10 °C) for 2 h for 1, 2, or 3 d and returned to normal growth conditions. The plants were also subjected to a day of acclimatization at 4 °C before and after the treatment. Following treatment, plants were allowed to recover for 1 or 2 d at ambient temperature before seeds were collected for either RNA extraction or allowed to mature and then observed for defects in degreening.

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