The Arabidopsis C2H2 Zinc Finger INDETERMINATE DOMAIN1/ENHYDROUS Promotes the Transition to Germination by Regulating Light and Hormonal Signaling during Seed Maturation

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Seed development ends with a maturation phase that imparts desiccation tolerance, nutrient reserves, and dormancy degree. Here, we report the functional analysis of an Arabidopsis thaliana C2H2 zinc finger protein INDETERMINATE DOMAIN1 (IDD1)/ENHYDROUS (ENY). Ectopic expression of IDD1/ENY (2x35S:ENY) disrupted seed development, delaying endosperm depletion and testa senescence, resulting in an abbreviated maturation program. Consequently, mature 2x35S:ENY seeds had increased endosperm-specific fatty acids, starch retention, and defective mucilage extrusion. Using RAB18 promoter ENY lines (RAB18:ENY) to confine expression to maturation, when native ENY expression increased and peaked, resulted in the transition from lower abscisic acid (ABA) content and decreased germination sensitivity to applied ABA. Furthermore, results of far-red and red light treatments of 2x35S:ENY and RAB18:ENY germinating seeds, and of artificial microRNA knockdown lines, suggest that ENY acts to promote germination. After using RAB18:ENY seedlings to induce ENY during ABA application, key genes in gibberellin (GA) metabolism and signaling were differentially regulated in a manner suggesting negative feedback regulation. Furthermore, GA treatment resulted in a skotomorphogenic-like phenotype in light-grown 2x35S:ENY and RAB18:ENY seedlings. The physical interaction of ENY with DELLAs and an ENY-triggered accumulation of DELLA transcripts during maturation support the conclusion that ENY mediates GA effects to balance ABA-promoted maturation during late seed development.

INTRODUCTION

The presence of desiccated seeds as reproductive bodies in higher-plant lineages has introduced a maturation phase that imparts stress tolerance upon the seed and provides a mechanism for propagation through both time and space (Bewley and Black, 1994). Temporally, the progression from body plan establishment to resumption of seedling growth involves three phase transitions (Feurtado and Kermode, 2007). One exists in the transition from a mature differentiated embryo to a mature desiccated embryo (eventually surrounded by developed accessory structures, such as the seed coat and endosperm); this encompasses both reserve accumulation and desiccation as the seed becomes a mobile heterotrophic entity. A second occurs as seed dormancy is terminated and germination processes begin, culminating with the protrusion of the radicle (visible germination). A third transition occurs during seedling growth when light becomes a trigger for photomorphogenesis and an autotrophic existence resumes. However, these are not distinct phase changes and arise during a gradual series of events that occur through development. For example, as Angelovici et al. (2010) note, some of the transcriptional and metabolic changes associated with germination have already commenced during seed desiccation.

The hormone abscisic acid (ABA) promotes seed maturation events, such as the deposition of nutrient reserves, desiccation tolerance, and induction of dormancy (Feurtado and Kermode, 2007; Holdssworth et al., 2008). ABA is an absolute prerequisite for proper progression through maturation as demonstrated by immunomodulation of ABA in tobacco (Nicotiana tabacum) and maize (Zea mays) viviparous mutants (Phillips et al., 1997; White et al., 2000). Although such direct evidence is lacking in Arabidopsis thaliana, it is clear that a high ratio of ABA to gibberellin (GA) is an important determinant of maturation and dormancy (Karssen et al., 1983; Debeaujon and Koornneef, 2000; Okamoto et al., 2010). The role of GA during seed development is less obvious. Ectopic expression of a pea (Pisum sativum) GA 2-oxidase in Arabidopsis resulted in both early and late aborted seed (Singh et al., 2002), suggesting a minimal GA content is essential for normal seed development. However, uncovering the full extent of GA function during seed development is not a simple task. The requirement for GA in flower development and pollination, and conceivably embryogenesis, makes identifying what role GA plays during seed maturation, if any, challenging.

In Arabidopsis, four major regulators of seed maturation have been characterized: FUSCA3 (FUS3), ABSCISIC ACID INSENSITIVE3 (ABI3), LEAFY COTYLEDON1 (LEC1), and LEC2 (Nambara
Mutants of ab13, fus3, lec1, and lec2 are all severely affected in many aspects of maturation; phenotypes include intolerance to desiccation (ab13, fus3, and lec1), reduced storage protein synthesis (all four mutants), anthocyanin accumulation (fus3 and lec1), chlorophyll degradation (ab13), cotyledon misidentity (fus3, lec1, and lec2), and reduced seed dormancy (all four) (Holdsworth et al., 2008). Additional studies have further defined the intricate network relationships that these major regulators form, including redundant and hierarchical regulation (Kagaya et al., 2005; To et al., 2006). LEC2 and FUS3 also modulate ABA and GA metabolism (Curaba et al., 2004; Gazzarrini et al., 2004).

Germination in Arabidopsis, as with many species, is stimulated by cues such as light, cold, nitrate, and the process of after-ripening (Finkelstein et al., 2008). Light stimulation of germination is chiefly triggered through the red (R) and far-red (FR) light-perceiving PHYTOCHROME proteins PHYA and PHYB (Reed et al., 1994). Major regulators of PHY action include PHYTOCHROME-INTERACTING FACTOR (PIF; or PIF3-like [PIL]) proteins whose role is to negatively regulate PHY action (and vice versa) (Shin et al., 2009). During germination, PIL5 has been shown to upregulate GA catabolism and ABA metabolism (Shin et al., 2009). The CCCH zinc finger SOMNUS is also directly regulated by PIL5 and has been shown to upregulate GA catabolism and ABA synthesis while downregulating GA biosynthesis and ABA catabolism (Kim et al., 2008). A key process that occurs during the transition to germination, seemingly irrespective of how dormancy is broken, is an increase in the ratio of GA to ABA, through increased GA synthesis and ABA catabolism and activation or derepression of downstream GA signaling (Finkelstein et al., 2008).

The current GA signaling pathway is fairly straightforward. Key regulators include the GID1 GA receptors, the DELLA repressors, SLEEPY1 (SLY1) and SNEEZLY F-box proteins, and the O-linked N-acetylglucosamine transferase (GlcNAc) SPINDLY (SPY) (Peng et al., 1997; Silverstone et al., 1998, 2007; McGinnis et al., 2003; Nakajima et al., 2006; Ariizumi et al., 2011). In the absence of GA, the nuclear-localized DELLA proteins are growth repressing. To upregulate the GA response, bioactive GAs bind to a GID1 receptor (GID1A, B, or C in Arabidopsis), promoting its interaction with a DELLA protein. The GA-GID1-DELLA complex shows greater affinity toward SLY1, and DELLA is subsequently degraded by the 26S proteasome pathway (Griffiths et al., 2006; Willige et al., 2007). SPY, a negative regulator of GA signaling, may participate in this regulatory cascade by increasing DELLA activity through GlcNAc modification (Silverstone et al., 2007). In Arabidopsis, there are five DELLA proteins, RGA, GAI, RGA-LIKE1 (RGL1), RGL2, and RGL3, which show distinct but overlapping functionality (Sun, 2008). The molecular mechanism of DELLA action is poorly understood; however, two groups have made a major breakthrough regarding DELLA participation in light-regulated hypocotyl elongation. de Lucas et al. (2008) and Feng et al. (2008) demonstrated that the DELLA proteins interact with the DNA binding domain of PIFs (PIF3 and PIF4), thus blocking PIF function and rendering them unable to participate in the promotion of hypocotyl elongation and other skotomorphogenic (dark-growth) traits. Further information regarding DELLA functionality has come from recent articles identifying new protein interactors. These include a DELLA interaction with SCARECROW-LIKE3 (SCL3) and ALCATRAZ (ALC) in a GA regulatory context and the JASMONIC ACID ZIM-DOMAIN (JAZ) proteins, which are key repressors of JA signaling (Arnaud et al., 2010; Hou et al., 2010; Zhang et al., 2011).

In this article, we characterize AT5G66730, a 26S proteasome pathway (Griffiths et al., 2006; Willige et al., 2007) and a DELLA interaction with SCARECROW-LIKE3 (SCL3) and ALCATRAZ (ALC) in a GA regulatory context and the JASMONIC ACID ZIM-DOMAIN (JAZ) proteins, which are key repressors of JA signaling (Arnaud et al., 2010; Hou et al., 2010; Zhang et al., 2011).

ENY mRNA Levels Increase during Seed Maturation

Initial interest in IDD1/ENY arose from a study linking IDD1/ENY to secondary seed dormancy in canola (Brassica napus) (Fei et al., 2007). Further analysis of various published microarray data sets revealed expression of ENY was also associated with dormancy in seeds of the Arabidopsis ecotypes Landsberg erecta and Cape Verde Islands (Cadmian et al., 2006; Carrera et al., 2007). AtGenExpress Development and Hormone microarray data sets also reveal that expression of ENY in the Arabidopsis life cycle is highest during seed development (Schmid et al., 2005; Goda et al., 2008).

To quantitate ENY expression during seed development, particularly during maturation, ENY transcript abundance was measured using quantitative RT-PCR (qRT-PCR) in Columbia wild-type seed from 6 d postanthesis (DPA) to 18 DPA and also in mature harvested seed. Consistent with previously published microarray results, ENY increased in expression (>50-fold) over the course of seed development (Figure 1A). In addition to Columbia wild type, ENY expression was also monitored in transgenic lines modulating ENY expression. These were 2x3SS ectopic misexpression (ENY-ME), RAB18 promoter-driven ENY (RAB18:ENY), and a RAB18 promoter-driven artificial microRNA (amiRNA) line (RAB18:ENYami) that targets ENY for downregulation (greater detail on each transgenic line is discussed in subsequent sections). Consistent with the constitutive nature of...
the 35S promoter, ENY-ME displayed a relatively consistent high abundance of ENY transcripts throughout development. The 2x35S promoter produced 60 times more ENY transcripts than the native wild-type gene at 6 DPA, but the difference in expression was only 2-fold in mature harvested seed due to the increase in expression of the native gene. In RAB18:ENY, expression of ENY was identical to the wild type at 6 DPA. However, as the RAB18 promoter was activated, ENY expression increased in parallel with the native gene but at levels 6- to 17-fold higher. Expression from RAB18:ENY was comparable to that produced by 2X35S:ENY at 12 DPA but was higher during later time points. ENY transcript abundance in the RAB18 promoter-driven amiRNA genotype was identical with the native gene and RAB18:ENY at 6 and 9 DPA but gently decreased at later time points. At the harvested seed stage, ENY was 40-fold downregulated and was almost two orders of magnitude lower than in the wild type (Figure 1A).

To spatially localize ENY expression, we monitored activity of β-glucuronidase (GUS) driven by the ENY promoter (Figure 1B). At 3 DPA, GUS activity was observed in the chalazal endosperm region. By 6 DPA, activity was present in the endosperm and embryo. GUS staining continued to be apparent in the
endosperm and embryo throughout seed development and continued in the early imbibed seed. Activity decreased during germination, and in the fully germinated seed, GUS staining was strongest in the root tip and absent from the cotyledons. In 4-d-old seedlings, GUS was restricted to the vasculature of the cotyledons, the shoot apical meristem region, and the root. The darkest staining was observed in the root tip and in emerging lateral roots. By 8 d, activity in the shoot was restricted to newly emerged leaves (Figure 1B).

Ectopic Misexpression of ENY Delays Seed Maturation Events

To functionally characterize ENY, we generated 2x35S misexpression lines using the open reading frame (ORF) of ENY (AT5G66730) (hence named ENY-ME) and obtained publicly available T-DNA insertion lines. However, no reduction in expression of ENY in mature rosette leaves of homozygous T-DNA lines was observed using qRT-PCR. Thus, initial analyses were focused on ENY-ME lines. After seed imbibition and staining with ruthenium red, we observed defective mucilage extrusion in 42% of T1 transgenic lines (see Supplemental Figures 1 and 2 online). Subsequently, analyses of two independent homozygous lines (3 and 19) in the T3 and T4 generations revealed that ENY misexpression perturbed many developmental processes, including fertility, seed development, germination, and seedling establishment (see Supplemental Figures 3 and 4 online). However, because of the association of ENY expression with seed dormancy, we focused on the role of ENY in seed development and germination.

A significant delay in ENY-ME seed development was observed, especially with respect to senescence of the seed coat and depletion of the endosperm (Figures 2 and 3; see Supplemental Figure 5 online). An enlarged endosperm is especially evident at 8 DPA when in the wild type there were only three to five cell layers of endosperm remaining and the embryo was constrained into the folded U-shape. In ENY-ME, a large amount of endosperm remained and the embryo was not U-shaped (Figures 2A and 3). The seed coat of ENY-ME at 8 DPA was similar to Columbia wild type, except that the epidermal cell layer appeared larger; however, this may be partly due to artificial swelling of the mucilage during aqueous fixation. At 10 DPA, embryo sizes were similar but the endosperm in ENY-ME lines retained three to five cell layers, while in wild-type endosperm, development was mostly complete and had shrunk to one to two cell layers with a distinct endosperm aleurone layer appearing. The seed coat at 10 DPA was thicker in ENY-ME lines; this appeared to be due to the increased size of cells underlying the epidermal layer such as the endothelium cells (Figure 3). By 15 DPA, wild-type seed coat development was mostly complete; however, ENY-ME seed coats were still translucent with limited pigmentation (Figure 2A). The fresh weight (water content) of ENY-ME was increased, but on a dry weight basis, ENY-ME seeds were not significantly different from Columbia wild type (Figure 2B). Unlike Columbia wild type, the desiccation of ENY-ME seeds was abrupt; most of the fresh weight was lost between 18 and 20 DPA (Figure 2B). By the end of seed maturation, mature ENY-ME
The area of ENY-ME seeds, as measured by ImageJ, was consistently 1.5-fold larger than Columbia wild type (wt) (0.27 versus 0.18 mm², 0.30 versus 0.20 mm², and 0.26 versus 0.17 mm² at 6, 8, and 10 DPA, respectively). The increase in area was due to an increase in endosperm and seed coat size. At 6 DPA, cell numbers in the endosperm of ENY-ME totaled 185, while Columbia wild type totaled 160. At 8 DPA, increased ENY-ME endosperm size meant that the embryo was no longer constrained in the prototypical U-shape. At 10 DPA, significant endosperm still remained to be absorbed in ENY-ME, a process mostly complete in Columbia wild type. The seed coat was also noticeably thicker at 10 DPA; pink arrows represent the comparison between endothelium cells of ENY-ME and Columbia wild type. Ct, cotyledons; E, embryo axis; En, endosperm; Sc, seed coat; Si, siliques. Green arrow at 8 DPA represents a cell where mucilage has artificially expanded during aqueous fixation.

Figure 3. Delayed Endosperm Depletion during ENY Ectopic Misexpression.

The apparent delay in ENY-ME seed maturation prompted us to measure seed storage compounds in the mature seed. Total protein extracted from ENY-ME lines and Columbia wild-type seed grown at the same time showed an average decrease of ~21 and 27% in lines 3 and 19, respectively (see Supplemental Figure 7A online). Consistent with the increased endosperm size, several endosperm-specific fatty acids (Penfield et al., 2004) were increased in mature seeds of ENY-ME lines (see Supplemental Figure 7B online). Sugars, including Suc, Glc, raffinose, and stachyose, were also increased in ENY-ME line 19. A significant amount of starch was present in mature seeds of ENY-ME line 19 but not in mature Columbia wild-type seed (see Supplemental Figure 7C online). Mature Columbia seed does not usually contain starch since it accumulates only transiently in Arabidopsis seed development (Focks and Benning, 1998).

To identify changes in gene expression associated with ENY action, we performed a two-color microarray analysis comparing ENY-ME line 19 to Columbia wild type at 5 DPA, 10 DPA, and mature seed (see Supplemental Data Set 1 online). Differentially expressed gene (DEG) lists, containing both up- and downregulated genes, were identified using a false discovery rate (FDR) of 5% using a modified t test in Significance Analysis of Microarrays (SAM) and 1.5-fold change in expression (Tusher et al., 2001). Consistent with phenotypic observations and measurement of storage compounds, transcript profiles of ENY-ME were at a more juvenile stage than those of the wild type. For example, at 10 DPA, Gene Ontology (GO) terms overrepresented in the downregulated genes (at P ≤ 0.001) included alkaloid metabolism, flavonoid and lignin biosynthesis, and response to water deprivation (see Supplemental Data Set 1 online). Reduced expression of genes associated with these processes is consistent with the observed delays in seed coat development and delayed desiccation in ENY-ME seeds compared with Columbia wild type. However, in mature ENY-ME seeds, several GO terms overrepresented in the upregulated genes suggest ENY-ME seeds rapidly advanced through the desiccation process: response to ABA, desiccation, water deprivation, cold, salinity, galactose metabolism, and Pro biosynthesis. Thus, the overall effect of ENY misexpression is to delay seed maturation events, especially with respect to seed coat senescence and endosperm assimilation.

Induction of ENY in Seedlings Shifts Expression of GA Pathway Genes

The significant developmental perturbations in ENY-ME lines during seed development meant that direct comparisons of tissues of the same age could not reveal the precise effects of ENY. Differences in patterns of gene expression could reflect shifts in timing of development rather than the direct effects of ENY on gene expression. Therefore, it was decided to direct expression specifically to seed maturation using the RAB18 promoter. We anticipated that the RAB18 promoter would direct expression in a similar temporal/developmental pattern as ENY but at a higher level (as confirmed in Figure 1A). RAB18 is also spatially localized throughout the embryo and endosperm in dry seeds (Nylander et al., 2001); this parallels ENY promoter activity as shown by GUS staining (Figure 1B). Therefore, RAB18:ENY transgenic lines should reveal the true function of ENY during seed maturation more precisely than the 2x35S lines. Since the RAB18 promoter is not expressed during early seed development, some of the confounding effects due to delayed entrance into seed maturation would be avoided. Accordingly, the seed phenotypes described for the ENY-ME plants described above (e.g., delayed seed coat senescence and mucilage defect) were absent in the RAB18:ENY plants (see Supplemental Figure 8 online). This suggests that the effects in ENY-ME lines are initiated early in seed development.
before ENY is normally expressed at a high level. However, as described below, other ENY-ME phenotypes were recapitulated in RAB18:ENY.

Having created transgenic RAB18:ENY lines to investigate high level expression effects during late seed development, we subsequently used these lines in an inducible system to determine the short-term effect of increased ENY expression on the Arabidopsis transcriptome. To accomplish this, RAB18:ENY seedlings were treated with 10 μM (+)-ABA to induce ENY (Ghassemian et al., 2000). After obtaining three independent T2 RAB18:ENY lines, 5-d-old seedlings were selected to perform the inductions, since the seedlings were easy to manipulate and expression of the RAB18 promoter under control conditions was minimal. Initial testing, using qRT-PCR expression analysis, showed that maximal expression of ENY occurred 3 h after transfer of the seedlings to (+)-ABA (see Supplemental Figure 9A online). It is also important to note that expression of ENY was not affected by ABA treatment in Columbia wild type (see Supplemental Figure 9B online). Nonetheless, knowing that the presence of ABA would alter seedling physiology, a transgenic ENY line was always compared with like-treated Columbia wild type (i.e., 3 h ABA-treated RAB18:ENY compared with 3 h ABA-treated Columbia wild type).

Two representative RAB18:ENY transgenic lines (2 and 16) displayed increases in ENY expression of 5-fold at time 0 and 73- and 57-fold after 3 h (+)-ABA, respectively (see Supplemental Figure 9A online). We compared RAB18:ENY to Columbia wild type at both time 0 (No ABA) and after 3 h with ABA (3 h ABA) (RAB18:ENY results were averaged between lines 2 and 16). There were only 22 DEGs in the No ABA comparison (see Supplemental Data Set 2 online). Notable in this list was downregulation of the GA biosynthesis gene GA 20-oxidase 2 (GA20ox2) in RAB18:ENY lines (see Supplemental Data Set 2 online). After treatment with ABA and subsequent ENY induction (3 h ABA), there were 221 DEGs (43 upregulated/178 downregulated). Several additional GA metabolism or signaling genes were present in the 3-h ABA DEG list (significantly changed ±1.5-fold). The catabolic genes GA2ox6 and GA2ox1 were upregulated, and the biosynthesis genes GA20ox2 and GA3ox1, the GA receptor GID1B, transcriptional regulator SCL3, and F-box SLY1 were downregulated (Figure 4A; see Supplemental Data Set 2 and Supplemental Figure 10 online).

We used qRT-PCR to further investigate the impact of ENY expression on GA metabolism and signaling. In this experiment, in addition to inducing ENY with 3 h (+)-ABA treatment, either 10 μM GA3 or 1 μM paclobutrazol (PAC; an inhibitor of GA biosynthesis) was included (see Supplemental Figure 9C online). The induction of ENY was similar in the various treatments with the exception of RAB18:ENY line 13 under ABA/GA (195-fold upregulated in ABA/GA versus 235 in the other two treatments, ABA or ABA/PAC) (see Supplemental Figure 9C online). As in the microarray results, increased expression of ENY resulted in upregulation of negative components of GA metabolism or signaling and downregulation of positive components of GA metabolism or signaling (Figure 4B). The largest changes in expression were observed under the ABA/GA treatment in the highest-expressing RAB18:ENY line, line 13. For example, comparing line 13 to Columbia wild type after 3 h ABA treatment, major changes in GA20ox2, GA3ox1, and GA2ox1 expression were observed (fold change of −4.0, −3.0, and 8.2, respectively). Under ABA/GA treatment, the expression of the aforementioned genes changed, −15.3-, −17.6-, and 15.7-fold, respectively. The effect of PAC combined with ABA produced expression changes that were close to those observed for ABA alone (Figure 4B). It was expected that PAC application would reduce the negative transcriptional impacts of ENY on GA homeostasis. However, this may have been masked due to the application of ABA or simply the presence of ENY itself. Nevertheless, these results associated ENY with GA metabolism and signaling. The next step in assigning a function to ENY was to investigate its possible association with other components of the GA pathway.

ENY Is a Negative Regulator of Photomorphogenesis in the Presence of GA

Following germination, Arabidopsis seedlings exposed to light undergo photomorphogenesis, which is characterized by reduced hypocotyl elongation, cotyledon opening and expansion, production of chlorophyll, and resumption of photosynthesis. In the dark, skotomorphogenic (or etiolated) seedlings have long hypocotyls, closed cotyledons in an apical hook, and contain etioplasts instead of chloroplasts. Etiolated growth is driven by GA and reducing endogenous GA levels partially derepresses photomorphogenesis in the dark (Alabadi et al., 2004). Thus, because results from the induction experiments seemed to associate ENY with GA homeostasis, we investigated the effect of ENY on seedling growth with and without addition of exogenous GA.

After 7 d growth in white light, the hypocotyl length and cotyledon angle of ENY-ME lines were similar to Columbia wild type when grown on control plates with 0.5 Murashige and Skoog (MS) salts (Figure 5). However, when grown in the light with 10 μM GA3, ENY-ME lines displayed hypersensitive effects. ENY-ME hypocotyl lengths were more than double the length of Columbia wild type (Figure 5A). In addition, cotyledon angles of ENY-ME seedlings grown in the presence of GA were smaller than their wild-type counterparts (Figure 5A). The hypocotyl effect was increased slightly when 1% Suc was added to the medium (see Supplemental Figure 11 online). These phenotypes, together with the presence of shorter roots and chlorotic, yellow cotyledons and leaves (Figure 5B; see Supplemental Figure 11B online), resemble dark-growth (skotomorphogenic) phenotypes. However, unlike etiolated seedlings, cotyledon expansion and activation of the shoot apical meristem were not appreciably affected. We classified the skotomorphogenic-like phenotype of light-grown ENY-ME lines as GA-hypersensitive (1) because of the intimate association of GA with etiolated growth (Alabadi et al., 2004) and (2) because the phenotype was associated with exogenous GA application. A similar GA-hypersensitive phenotype was also observed in RAB18:ENY seedlings when grown under 10 μM GA3 and 0.45 μM (+)-ABA to induce ENY, but only with respect to increased hypocotyl elongation (see Supplemental Figure 12 online). A mild phenotype was not unexpected, however, as ENY expression would not be sustained under the RAB18 promoter, partly because ABA was applied at a low concentration and would be rapidly metabolized (Huang et al., 2007).
ENY Promotes Phytochrome-Mediated Seed Germination

Next, we investigated the effect of ENY overexpression during early germination. After confirming that ENY was differentially expressed in dry and imbibed seeds of RAB18:ENY lines (see Supplemental Figure 13A online), we performed microarray analyses comparing RAB18:ENY line 13 to Columbia wild type after 1 and 3 h in germination conditions under white light (WL) (see Supplemental Data Set 3 online). The comparison yielded 945 DEGs (241 upregulated/704 downregulated) after 1 h seed imbibition and 735 DEGs (229 upregulated/506 downregulated) after 3 h seed imbibition. Consistent with the previous ENY induction experiment results, GA biosynthesis and signaling were repressed while GA catabolism and negative regulatory factors were activated (Figure 4A; see Supplemental Data Set 3 online).

In a recent study, Oh et al. (2009) suggested ENY is a direct downstream target for regulation by PIL5. Based on this information and our association of ENY with the inhibition of photomorphogenesis, we compared the DEGs in our data set, comparing RAB18:ENY to Columbia wild type after 1 h in germination conditions, with two data sets available online from the Gene Expression Omnibus (GEO) archive (Barrett et al., 2009). The first study, from Oh et al. (2009), compared Columbia wild-type imbibed seed treated with FR and red R light pulses to that treated with a FR light pulse alone (after a subsequent 12 h in the dark), hence named Col-0(D)/Col-0(R). Treatment with an R light pulse stimulates phytochrome-mediated germination of Columbia wild type seeds in the dark. Thus, in the FR/R treatment, the transcriptional profiles would be those of a germination-stimulated nature. A considerable portion of DEGs in our RAB18:ENY/Columbia data set overlapped with the Col-0(R)/Col-0(D) DEGs (Figure 6A; see Supplemental Data Set 3 online). For example, 45% of genes downregulated by the presence of ENY at 1 h white light were also downregulated by red light after 12 h; whereas 28% were commonly upregulated by both ENY and red light. The second experiment, from Leivar et al. (2009), compared the quadruple pifq mutant (pif1 3 4 5) and Columbia wild type after 5 d of moist chilling, hence named pifq(seed)/Col-0(seed). As mentioned, PIFs are negative regulators of phytochrome action, and the pifq mutant, because of the lack of the PIL5/PIF1 protein, can germinate in the dark without the need for a R light cue (Shin et al., 2009). Similar to the comparison to R light, substantial overlap occurred between the RAB18:ENY/Columbia and the pifq(seed)/Col-0(seed) comparison (Figure 6A; see Supplemental Data Set 3 online). Here, 36% of the genes downregulated by
the presence of ENY at 1 h in germination conditions were also downregulated by PIF absence, whereas 19% were commonly upregulated by increased ENY presence and PIF absence. Thus, ENY regulated, perhaps indirectly, a high proportion of genes modulated by both red light and PIFs, in the same direction as red light but opposite to the action of PIFs.

After finding substantial overlap between ENY-regulated transcription, red light, and PIFs, we tested the germination response of ENY mis- and overexpression lines to both FR and R light. We focused on FR and R light treatment early in the imbibition process since two-thirds of ENY transgenic lines were driven by the RAB18 promoter and expression from this promoter is greatly reduced within the first 5 h of imbibition (see Supplemental Figure 13A online). Because FR and R light were applied during the first 1 to 2 h of imbibition, PHYB, mediating the R light low fluence response, would be expected to be the dominant phytochrome promoting germination at this time (Reed et al., 1994; Shinomura et al., 1994). Following plating of seeds under

Figure 5. Growth of ENY Misexpression Seedlings under GA Treatment Results in a Skotomorphogenic-Like Phenotype.

(A) Hypocotyl length and cotyledon angle of ENY-ME and Columbia wild-type (Col wt) 7-d-old seedlings grown with or without GA$_3$. Error bars represent the SE of at least 50 measured seedlings.

(B) Seedling phenotype under GA treatment of ENY-ME seedlings as detailed in (A). Bar = 5 mm.

Figure 6. RAB18 ENY Array Data Display Similarities to Red Light, the Quadruple pif Mutant, and GA-Treated ga1-3 Data Sets.

(A) Overlap of genes between RAB18:ENY/Columbia wild type 1 h imbibed seed array data and the data of Oh et al. (2009) comparing FR light/FR-R light 12 h after the start of seed imbibition.

(B) As in (A) but comparison of our data to Leivar et al. (2009) data comparing pif1 3 4 5 quadruple mutant seed to Columbia wild type seed moist-chilled for 5 d.

(C) Comparison of genes changed ≥2-fold in RAB18 ENY knockdown and overexpression 1 h imbibed seed microarray data sets (see Supplemental Data Sets 3 and 4 online) with Oh et al. (2009) data set described in (A) and Ogawa et al. (2003) data set. The Ogawa et al. (2003) data set compared ga1-3 seed treated with GA for 9 h to ga1-3 treated with water for 9 h.
white light for 1 h, seeds were treated with a pulse of 4 μmol/m²/s FR for 6 min. Consistent with the observed changes in transcriptional events in RAB18:ENY, both ENY-ME and RAB18:ENY lines were less sensitive to germination inhibition by FR light (see Supplemental Figure 14 online). To stimulate PHYB-mediated germination after the white light and FR treatment, seeds received 30 μmol/m²/s R light for 10 min. Here, FR/R light treatment promoted germination to virtually 100% in both ENY lines and Columbia wild type (see Supplemental Figure 14 online). Thus, ENY alters the behavior of Arabidopsis seeds so that they are less dependent on light-triggered cues for germination.

Knockdown of ENY in Seeds Supports a Role in Light and Hormonal Signaling

Since no T-DNA insertion lines resulted in differences to ENY expression, amiRNA methodology was used to attenuate (knockdown) ENY expression (Schwab et al., 2006). As before, for a high level of seed-specific expression during seed maturation, we used the RAB18 promoter to drive amiRNA expression, and this resulted in a reduction of 96 to 98% in mature T3 homozygous seeds (see Supplemental Figure 13B online).

Because ENY-ME and RAB18:ENY lines displayed altered FR and R light germination, we tested the response of RAB18:amiENY lines under similar conditions. Opposite to ENY overexpression lines, knockdown lines were less sensitive to germination promotion by R light compared with Columbia wild type (see Supplemental Figure 14 online). Since RAB18:amiENY line 24 showed the greatest phenotypic germination response and largest ENY knockdown, we used this line for microarray experiments.

For uniformity with the RAB18:ENY data sets, we compared RAB18:amiENY line 24 and Columbia wild type after 1 h in germination conditions under white light. Using the same criteria as in previous experiments, 620 DEGs (392 upregulated and 228 downregulated) were identified in the RAB18:amiENY to Columbia comparison (see Supplemental Data Set 4 online). Unlike the results of the RAB18:ENY arrays, few GA synthesis or signaling genes were revealed. In fact, only GA2ox6 was present within the DEG lists and was downregulated (GA2ox6 was significantly [1.44-fold] upregulated). However, several factors involved in light and phytochrome signaling networks were altered (see Supplemental Table 1 online). For instance, inhibitors of photomorphogenesis were upregulated, such as CONSTITUTIVE PHOTOMORPHOGENIC10 (COP10)/FUS9 and SUPPRESSOR OF PHYA 1-LIKE3 (SPA3) (Suzuki et al., 2002; Laubinger and Hoecker, 2003). Other factors involved in light signaling that were downregulated in the knockdown line included the downstream PHYA factor LONG AFTER FAR-RED3 (LAF3) and the shade avoidance–related factor ARABIDOPSIS THALIANA HOME-BOX PROTEIN2 (ATHB2) (PHYA itself was significantly [1.33-fold] downregulated) (Steindler et al., 1999; Hare et al., 2003). Another trend in RAB18:ENYami DEGs was the upregulation or derepression of genes involved in photosynthesis and associated processes. Four subunits of RUBISCO, 14 genes involved in the photosynthetic light reactions, and two enzymes in the photosynthetic pathway were upregulated in ENY knockdown seed (see Supplemental Data Set 4 online). The derepression of photosynthetic processes, even after 1 h in germination conditions, is consistent with a role for ENY in skotomorphogenic-like signaling.

The expression of the two GA metabolic genes, GA2ox6 and GA20ox3, in the ENY knockdown line was opposite to that observed in the RAB18:ENY overexpression line, further suggesting a consistency in the phenotypes. However, when comparing the entire data sets, only 71 DEGs were oppositely regulated in RAB18:ENY and RAB18:amiENY comparisons (see Supplemental Data Set 4 online). Seventeen DEGs were upregulated in RAB18:ENY and downregulated in RAB18:amiENY, and 54 were common in the opposite direction (downregulated in RAB18:ENY and upregulated in RAB18:amiENY) (see Supplemental Data Set 4 online). A deeper comparison with ENY RAB18 overexpression and knockdown gene lists revealed that several genes involved in light or hormonal signaling were significantly regulated in the opposite direction (see Supplemental Table 1 online). For instance, in the ABA signaling pathway, the negative regulator ABI1 was upregulated with ENY overexpression and downregulated when ENY expression was knocked down. Similarly, GNC (for GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED), which was recently identified as a repressor of GA responses (Richter et al., 2010), was upregulated in the RAB18:ENYami knockdown line and downregulated in the ENY-ME and RAB18:ENY overexpression lines (see Supplemental Data Set 3 and Supplemental Table 1 online).

As a final comparison for the RAB18:ENY and RAB18:amiENY data sets, we examined how DEGs in our data sets changed during red light and GA treatment. To accomplish this, we used the Col-0(R)/Col-0(D) data from Oh et al. (2009) mentioned above and an additional GEO microarray data set, from the AtGen-Express consortium, comparing ga1-3 seed treated with 5 μM GA4 to ga1-3 treated with water after 9 h in white light, hence named ga1-3(GA)/ga1-3(water) (Ogawa et al., 2003; Goda et al., 2008). After creating a virtual GeneSpring experiment with the red light and ga1-3 data expressed as log2 ratios, we imported RAB18:ENY and RAB18:amiENY’s gene lists and found that DEGs with ≥2-fold change showed the clearest trend. It is evident in Figure 6C that genes upregulated by ENY overexpression had a tendency to be upregulated by both red light and GA, while genes downregulated by ENY overexpression displayed the opposite trend (Figure 6C). The trend is modest, but still evident and opposite, in the RAB18:ENYami knockdown line. Genes downregulated by ENY knockdown were normally upregulated by red light and/or GA; genes upregulated when ENY was knocked down tended to be downregulated by red light and/or GA (Figure 6C). These results are consistent with the premise that ENY acts as a positive regulator of phytochrome and/or GA action.

ENY Antagonizes ABA Accumulation and Response

Having associated ENY with phytochrome and GA signaling, attention was shifted to ABA. Antagonism of GA and ABA has been documented from the earliest of studies in Arabidopsis; a key example was obtained when the ABA-deficient mutant aba1 was recovered in a suppressor screen for the GA-deficient ga1 mutant (Koornneef et al., 1982). To assess response to ABA, we tested the various ENY transgenic lines for sensitivity of germination to exogenous ABA. Consistent with the GA-hypersensitive
phenotype of ENY-ME seedlings. ENY-ME and RAB18:ENY transgenic lines were less sensitive to the inhibition of germination by applied ABA. Of note, ENY-ME lines germinated slower than Columbia wild type on water with or without prior moist chilling (Figure 7A; see Supplemental Figure 15 online); this may be related to the mucilage defect present in ENY-ME lines (Arsovski et al., 2009). Despite the delay in germination, the insensitivity of germination to ABA in ENY-ME lines was revealed at 1 and 10 μM (+)-ABA (Figure 7A; see Supplemental Figure 16 online). In contrast with the mis- and overexpression lines, RAB18:ENYami knockdown line 24 was more sensitive to germination inhibition by exogenous ABA (Figure 7A). If the light intensity was lowered from 90 to 40 μmol/m²/s, line 21 also displayed greater sensitivity to exogenous 0.5 and 1 μM (+)-ABA compared with Columbia wild type (see Supplemental Figure 16 online). Maintaining the consistency of ENY as an antagonist to ABA, mature seeds of both ENY-ME and RAB18:ENY lines contained lower amounts of endogenous ABA compared with Columbia wild type (Figure 7B).

**ENY Physically Interacts with the DELLA Protein Family**

In order to better define the functional context of ENY, we performed a yeast two-hybrid protein–protein interaction analysis. To that end, a commercially available Arabidopsis library from Clontech was screened for ENY interactor. The premade Clontech library was constructed with normalized cDNA from 11 different Arabidopsis tissues (see Methods). Initially, the ORF for ENY was cloned into the pGKT7 vector. However, expression of ENY with a 706-bp fragment of the Saccharomyces cerevisiae ADH1 promoter led to activation of all transcriptional reporters/ selectable markers (often termed autoactivation). To avoid this problem, we expressed ENY under a weaker promoter; the vector pGBT9, containing a 396-bp ADH1 promoter, was used for this purpose. Using pGBT9, autoactivation was reduced to minimal levels on the highest stringency plates (Figure 8A).

Following screening of >11 million yeast library clones through diploid mating, ~300 blue colonies of potential ENY interactors were recovered. After sequencing 90 of the potential ENY interactors, four DELLA proteins were recovered (RGL1 twice, RGL2, and RGA). RGL2 and RGA were in frame with the GAL4 activation domain (AD) but were lacking the DELLA protein domain. The two RGL1 clones were not in frame with the GAL4-AD but contained the DELLA domain; inclusion of the DELLA domain may have promoted transcriptional activation without the need of the GAL4-AD (de Lucas et al., 2008). We then proceeded to reverify the interactions in yeast by cloning the full-length ORFs of all five DELLA in frame with the GAL4-AD in the yeast vector pGADT7. All five DELLA proteins showed strong interactions with ENY on both low (plasmid selection only) and high (selecting for the protein:protein interaction) stringency plates containing the chromogenic marker X-α-Gal (Figure 8A). Quantification of the ENY-DELLA interaction using a β-galactosidase activity assay verified strong interactions compared with the negative controls (see Supplemental Figure 17A online).

To independently verify the interaction of ENY with each DELLA protein, we performed bimolecular fluorescence complementation (BiFC) assays in Nicotiana benthamiana leaf epidermal cells. Before attempting BiFC assays, the subcellular localization of yellow fluorescent protein (YFP)-ENY was confirmed by transient expression in N. benthamiana leaf epidermal cells. Consistent with previous reports on the IDD family (Welch et al., 2007; Wong and Colasanti, 2007), eYFP-ENY was nuclear localized (see Supplemental Figure 18 online). Since the GAL4 fusions in the yeast two-hybrid experiment were on the N terminus of the protein, the amino acids 1 to 174 of YFP were fused to the N terminus of ENY. Similarly, the amino acids 175 to 239 of YFP were fused to the N terminus of each of the DELLA proteins. The highest fluorescence in the negative controls occurred when the two halves of YFP were both fused to ENY; however, this signal was very weak compared with the YFP1,174-ENY and YFP175-end DELLA signals. All ENY-DELLA interaction combinations showed high and consistent fluorescent signals except GAI; however, GAI was still slightly higher than background fluorescence (Figure 8B; see Supplemental Figure 17B online). The interaction of ENY with DELLA proteins, via both the yeast two-hybrid and BiFC approaches, demonstrated a mechanistic and functional context for ENY: that DELLA proteins may modulate ENY function and/or that ENY may modulate DELLA function.

**ENY Modulates the Expression of DELLA and SCL3 during Seed Development**

To further investigate the connection of ENY and DELLA function, we measured DELLA expression during seed development in Columbia wild type and ENY transgenic lines (Figure 9). First, RGL1 and RGL3 expression levels were similar between Columbia wild type and all ENY transgenic lines. In addition, DELLA expression in Columbia wild type and the RAB18:ENYami knockdown line was generally similar. However, there were clear differences in GAI, GAI, and RGL2 in ENY-ME and RAB18:ENY transgenic lines. In the ENY-ME line, GAI levels increased sharply between 9 and 18 DPA and were still slightly elevated in mature harvested seed. In the RAB18:ENY line, increased GAI levels were seen only from 15 DPA onwards, somewhat paralleling the later increase in ENY seen in the RAB18 lines (Figures 1 and 9). Both GAI and RGL2 expression increased during 12 to 18 DPA in both ENY-ME and RAB18:ENY, and RGL2 was still elevated >2-fold in mature harvested seed in both ENY-ME and RAB18:ENY lines.

Previous studies identified SCL3 as a GA-repressed and DELLA-induced transcript (Zentella et al., 2007). More recently, SCL3 function has been further defined to include a positive regulatory role in GA signaling, including an SCL3 and DELLA antagonism and role in root cell elongation and ground tissue maturation (in a regulatory pathway defined with SHORTROOT [SHR], SCARECROW [SCR], and DELLA) (Heo et al., 2011; Zhang et al., 2011). Since SCL3 was downregulated in the RAB18:ENY induction experiment (Figure 4; see Supplemental Data Set 2 online), we profiled its expression during seed development. In Columbia wild type, SCL3 expression increased during seed development and was highest in the mature harvested seed (Figure 9). In ENY-ME, SCL3 abundance was lower than Columbia wild type between 6 and 12 DPA but rose to wild-type levels from 15 DPA to mature harvested seed (paralleling the increase in DELLA GAI and RGL2). RAB18:ENY, on the other
hand, was similar to the wild type at 6 and 9 DPA, when the RAB18 promoter was not as active, and then dropped to considerably lower levels than Columbia wild type through the rest of seed development and also in the mature harvested seed. The RAB18:ENYami line displayed the opposite trend at 15 and 18 DPA; SCL3 levels were higher than Columbia wild type (Figure 9).

**DISCUSSION**

Only four members of the Arabidopsis IDD family have been linked to a physiological role. Three members, NUTCRACKER/IDD8 (NUT), MAGPIE/IDD3 (MAG), and JACKDAW/IDD10 (JKD), are involved in a regulatory loop with the GRAS domain proteins SHR and SCR associated with Arabidopsis root development and patterning. SHR has been shown to directly regulate NUT and MAG expression, while MAG and JKD have been shown to physically interact with SHR and SCR (Levesque et al., 2006; Cui et al., 2007; Welch et al., 2007). Recently, Seo et al. (2011) found that NUT/IDD8 regulates the transition to flowering through its regulation of sugar metabolism. SHOOT GRAVITROPISM5/IDD15 (SGR5) has been linked to gravity perception and starch accumulation in inflorescence stems (Tanimoto 1782. The Plant Cell
et al., 2007). While ENY to regulate SGR5 mutant, which has a one amino acid deletion in ENY causes feedback downregulation of GA homeostasis. We were confronted with an apparent inconsistency in ENY function that there was (1) a positive relationship to GA signaling through FR/R light germination and hypocotyl growth experiments versus (2) the opposite negative regulatory role of ENY on the expression of GA metabolism and signaling genes. Two possibilities were considered: (1) ENY promotes GA-associated downstream signaling events (e.g., expression of EXPA1) but at the same time inhibits upstream GA synthesis and signaling (e.g., expression of GA3ox1), or (2) ENY promotes GA-associated downstream signaling events and, because of this (as a secondary effect), feedback regulation is engaged to counteract perceived increases in GA signaling. After reviewing the literature on GA synthesis and signaling, it became evident that the second hypothesis was more likely.

Feedback regulation of GA homeostasis has been well documented. For example, GA3ox and GA20ox expression was elevated in GA biosynthetic mutants and decreased by GA treatment in the mutants or wild type (Cowling et al., 1998; Xu et al., 1999). By contrast, GA20ox expression was decreased in the ga1-2 biosynthetic mutant and increased upon GA treatment (Thomas et al., 1999). More recently, when RGA was induced via a glucocorticoid-dexamethasone-inducible system in seedlings, the GA biosynthesis genes GA3ox1 and GA20ox2 and GA receptors GID1A and GID1B were upregulated. One hour of GA treatment in 8-d-old ga1-3 seedlings resulted in downregulation of these two biosynthetic genes and two GA receptor genes (Zentella et al., 2007). Thus, applied GA or mutations that increase GA signaling (e.g., gai-t6 and rga-24) result in negative feedback regulation. Inhibition of GA synthesis or mutations that decrease GA signaling (e.g., gai) result in positive feedback regulation of GA homeostasis (Sun, 2008).

Consistent with the theme of ENY feedback regulation, five (GA4, GA20ox2, SCL3, AT4G19700, and GID1B) of the top six genes that Zentella et al. (2007) identified as GA downregulated and DELLA upregulated were also downregulated by ENY during induction experiments (see Supplemental Data Set 2 online). Additional observations suggesting feedback regulation were the changes in the upstream GA biosynthesis genes, GA2 and GA3, and in other downstream genes, such as GA3ox2 (GA4H), that have not previously been reported to be involved in feedback regulation (Yamaguchi et al., 1998; Helliwell et al., 1998; Matsushita et al., 2007). GA2 and GA3 were downregulated in imbibed seeds of RAB18:ENY line 13, and GA3ox2 was downregulated when ENY was induced with ABA with even greater repression under ABA/GA treatment compared with Columbia wild type (Figure 3B; see Supplemental Table 1 online).

Matsushita et al. (2007) isolated a protein, AT-HOOK PROTEIN OF GA FEEDBACK1 (AGF1), which bound to a cis-acting DNA motif, GNFEI, named for its role in the GA feedback mechanism. Overexpression of AGF1 promoted the positive feedback loop (upon inhibition of GA synthesis) and counteracted the negative loop (upon GA treatment). Interestingly, expression of AGF1 is downregulated 2-fold in 3-h imbibed seeds of RAB18:ENY line 13 (see Supplemental Data Set 3 online). This suggests that the presence of ENY acted to curtail the modulation of feedback
through AGF1 (i.e., the plant wanted greater negative feedback inhibition of GA homeostasis, so AGF1 was downregulated).

To our knowledge, increased ENY transcription resulted in one of the most comprehensive changes in the GA signaling pathway reported in the literature; the only major signaling factor that was not changed in any of our experiments, if we consider redundancies in gene families, was SPY. In tobacco, the basic Leu zipper REPRESSION OF SHOOT GROWTH (RSG) has been found to repress accumulation of GA through regulation of ent-kaurene oxidase and negative feedback regulation of GA20ox1 (Fukazawa et al., 2010). In rice (Oryza sativa), YABBY1 is also involved in negative feedback regulation of GA3ox2 (Dai et al., 2007). Feedback regulation may be related to ENY’s interaction with DELLAs proteins; the consequences of this antagonistic interaction are discussed below.

Figure 9. DELLAs and SCL3 Expression Is Altered by ENY Overexpression during Seed Development.

Expression of RGL2, RGA, and GAI was markedly altered when ENY expression was increased using the 2x35S or RAB18 promoters (ENY-ME and RAB18:ENY, respectively). By contrast, DELLAs expression during ENY knockdown (RAB18:ENYami) was similar to that of the Columbia wild type (wt). SCL3 expression displayed opposite trends compared with the DELLAs. SCL3 was downregulated in ENY-ME and RAB18:ENY lines. However, in ENY-ME, SCL3 expression returned to wild-type levels during the time when RGL2 and GAI increased. Error bars represent the SE of two biological replicates. Data were normalized to 10^4 copies of UBC21. Transgenic lines used for analysis included ENY-ME line 3, RAB18:ENY line 13, and RAB18:ENYami line 24. MS represents mature harvested seed.

Ectopic ENY Overexpression Delays Seed Maturation Events

Several lines of evidence suggest ENY modulates and counteracts the maturation process regulated by ABA (Santos-Mendoza et al., 2008). First, 35S misexpression lines exhibit delayed maturation processes, particularly senescence in the seed coat and endosperm. This phenotype seems related to upregulation of ENY during early seed development. It was not observed in ENY lines driven by the RAB18 promoter, which increased ENY expression above wild-type levels much later in seed development when events such as endosperm assimilation were almost complete (Figure 1; see Supplemental Figure 8 online). Although the ENY-ME phenotype is unique, it has similarities to fus3, abi3, lec2, and lec1 mutants (Nambara et al., 1992; Keith et al., 1994;
ENY-ME lines, seed desiccation was delayed and occurred abruptly; this is reminiscent of the desiccation intolerance of fus3, abi3, and lec1 mutants. However, as in weak abi3 and lec2 mutants (Ooms et al., 1993; Stone et al., 2001), the majority of ENY-ME seed were tolerant to desiccation at maturity. The second resemblance to fus3, abi3, lec2, and lec1 mutants was the accumulation of lower and altered levels of storage compounds. ENY-ME lines had reduced levels of protein reserves and increased accumulation of starch and endosperm-specific fatty acids. FUS3 mutants, for example, also display increased levels of starch and lower protein content (Tiedemann et al., 2008). As starch is usually transitory in wild-type Arabidopsis (Focks and Benning, 1998), the presence of starch in mature ENY-ME seeds suggests that normal reserve partitioning was not completed before desiccation. Similarly, seed coat and endosperm development was delayed and, at least with respect to the seed coat, was not completed before seed desiccation. This is evidenced by the failure of mucilage to extrude from the seed coat upon imbibition and the presence of increased endosperm-specific fatty acids.

From changes in gene expression in ENY overexpressing seed (see Supplemental Data Set 1 and Supplemental Table 1 online), it can be seen that ENY downregulated the maturation-promoting factor FUS3, which was correspondingly upregulated in the ENY knockout line. However, the effects of ENY expression on FUS3 were moderate and confined to late maturation and early imbibition as no changes were detected at 10 DPA, a time when FUS3 is highly expressed. Nonetheless, ENY's regulation of FUS3 is consistent with ENY being a modulator of maturation and subsequent germination, as FUS3 has been found to repress GA synthesis and induce ABA production (Gazzarrini et al., 2004; Curaba et al., 2004). Conversely, ABI1, a negative regulator of the ABA response, was positively influenced by ENY (see Supplemental Data Set 1 and Supplemental Table 1 online). ABI1 is a member of a group of protein phosphatase type-2C proteins that are negative regulators of ABA-related responses (Yoshida et al., 2006). ABI1 family members, SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE2 (SnRK) proteins, and the recently discovered ABA receptor family of PYRABACTIN RESISTANCE (PYR)/PYR-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR proteins have been shown to interact directly in an ABA regulatory hub (Fuji et al., 2009; Park et al., 2009). PYL11 was in our DEG list in RAB18 ENY overexpression and knocked down lines and changed in parallel with ABI1. The precise role PYL11 may play in ABA signaling has not been revealed (Park et al., 2009).

ABA accumulation was also reduced in mature seeds of ENY overexpression lines, and ABA sensitivity was negatively associated with ENY transcription (i.e., ENY overexpression lines were ABA hypersensitive, and knockout lines were hypersensitive compared with Columbia wild type) (Figure 7; see Supplemental Figure 16 online). These data suggest ENY acts antagonistically to ABA; in view of the direct relationship between ABA and seed maturation, it is consistent that ENY negatively affects maturation.

ENY Promotes Germination and Selectively Regulates Growth-Associated Factors

Evidence for positive regulation of germination by ENY is that ENY-ME and RAB18-ENY seed germination is insensitive to FR light (see Supplemental Figure 14 online), that RAB18-ENYami seed germination is FR hypersensitive and R insensitive (see Supplemental Figure 14 online), and gene expression profiles are consistent with ENY mediating red light and GA signaling (Figure 6). Given the skotomorphogenic-like phenotype under GA treatment, the ENY-DELLA interaction, and feedback-like effects on GA homeostasis, ENY seems to be related to GA and growth-associated signaling. Moreover, ENY also repressed expression of the GATA-type transcription factor GNC (expression was decreased in ENY-ME and RAB18-ENY and increased in the RAB18:ENYami knockdown) (see Supplemental Table 1 online). GNC expression is repressed by GA application and elevated in ga-1 and gid1abc mutants. GNC and its homolog GNC-LIKE function as repressors of GA action, inhibiting germination and elongation growth and promoting greening (Richter et al., 2010). A close association of ENY with the GA pathway is also consistent with the fact that phytochrome functions to activate GA synthesis during germination but attenuates GA action during photomorphogenesis (Yamaguchi et al., 1998; Achard et al., 2007; Feng et al., 2008; Strasser et al., 2010).

In addition to GA, ENY also regulated the brassinosteroid pathway, which is known to be involved in the promotion of growth and cell elongation (see Supplemental Table 1 online). BAS1 and SOB7, whose activation-tagged mutants were recovered in suppressor screens for phyB, metabolize the brassinosteroids (BRs) brassinolide and castasterone to inactive products through hydroxylation (Turk et al., 2005). As suppressors for phyB, BAS1 and SOB7 negatively regulate hypocotyl elongation and cotyledon area (Turk et al., 2005). In RAB18:ENY imbibed seeds, both BAS1 and SOB7 were downregulated, consistent with ENY being a promoter of growth, in this case via BRs. BRs are also inactivated by glucosylation through the DOGT1 gene product (Poppenberger et al., 2005), which was downregulated in RAB18:ENY seeds. However, the biosynthetic gene BRASSINOSTEROID-6-OXIDASE2 (BR6ox2), which catalyzes the last committed steps to active brassinolide, was also downregulated in RAB18:ENY imbibed seeds; however, this could be the result of feedback inhibition. BR6ox2 was also downregulated during GA treatment of ga-1-3 seedlings (Zentella et al., 2007).

ENY Interacts with DELLA Proteins and Causes Feedback-Like Regulation of DELLA Transcripts during Seed Development

Integration of light and GA signaling was demonstrated by de Lucas et al. (2008) and Feng et al. (2008), who showed that DELLA and PIF directly interact to create a regulatory node that modulates plant growth. Both groups demonstrated how postgerminationative seedling growth in light and dark conditions is modulated by the DELLA-PIF interaction and revealed how DELLLas may exert their effects on transcription to restrain growth. The two groups demonstrated that the DELLA-PIF interaction blocked the transcriptional activation function of PIFs; thus, in the light, when GA
levels are low and DELLA concentrations are high, DELLA would abrogate PIF-promoted processes, such as cell elongation. The effects of PIFs are also blocked by interaction with activated Pfr phytochromes, such as PHYB, which subsequently tag the PIF for proteasomal degradation (Al-Sady et al., 2006; Shen et al., 2007). In the dark, without the activated PHYs to curtail their action, PIFs and associated factors promote GA accumulation; high GA levels promote the proteasome-mediated degradation of DELLA via the action of the GID1-SLY1SCF E3 ubiquitin ligase complex; thus, PIFs are released (Griffiths et al., 2006). The identification of additional DELLA interactors has also furthered our knowledge of how DELLA act as global repressors of development and growth. During silique development, ALC is involved in defining the separation layer responsible for fruit opening. It has been suggested that DELLA interaction with ALC blocks its action and, thus, separation layer formation (Arnaud et al., 2010). Hou et al. (2010) demonstrated that DELLA interact physically with JAZ1 to compete with and block the JAZ1-MYC2 interaction, thus allowing MYC2 to perform its functional role as an activator of JA responses. More recently, it was shown that SCL3 is a positive regulator of the GA response and that its interaction with DELLA modulates GA homeostasis, creating an antagonistic relationship (Zhang et al., 2011).

So how does ENY functionality fit within the context of DELLA function? Given the opposing regulatory effects of ENY and DELLA, their direct interaction suggests a similar antagonistic relationship to that of SCL3. Thus, ENY may block DELLA action by disrupting DELLA protein interactions, thereby allowing factors, such as PIFs, to function. Alternatively (or in addition), DELLA may inhibit ENY action. Future experiments will identify which genes ENY directly regulates and the importance of DELLA interaction to ENY function.

The increase of RGA, GAI, and RGL2 expression during seed development, particularly during late maturation, in ENY-ME and RAB18:ENY lines also suggests an ENY-DELLA antagonism. Given the results from the RAB18:ENY induction experiments that showed negative-feedback regulation of GA homeostasis, the increase in RGA, GAI, and RGL2 during seed maturation could suggest that the increase in ENY expression is being balanced by DELLA-mediated feedback effects. RGL2, RGA, and GAI are the main DELLA that have been identified as repressors of germination (Lee et al., 2002; Tyler et al., 2004; Piskurewicz et al., 2009). More specifically, RGL2, RGA, and GAI are involved in the repression of testa and endosperm rupture and promote the maintenance of endogenous ABA and ABIS levels in the imbibed seed (Piskurewicz et al., 2008, 2009). Recently, it was shown that an RGL2-dependent release of ABA in the endosperm helps regulate embryo growth in dormant seeds (Lee et al., 2010).

A role for DELLA in balancing ENY function and the strict negative-feedback inhibition on GA homeostasis that we observed may explain why alterations to ENY transcripion in over-expression lines did not produce stronger phenotypes. With respect to ENY knockdown lines, the subtle phenotypes observed can likely be attributed to the lack of complete knockout in ENY transcripion and the genetic redundancy created by other members of the ID family.

Integrating the evidence we have compiled, we present a model of ENY function during seed maturation and early germination (Figure 10). In the simplest terms, ENY functions to promote GA-associated responses and repress ABA responses (or rather, subsets of those responses). However, our observations suggest a more complex effect, which includes changes in other hormonal pathways. In addition, ENY may promote select...
maturational processes, such as the repression of photosynthetic events. Finally, although we propose that ENY is a positive regulator of growth, it also strongly downregulates GA synthesis and signaling. Thus, ENY creates a situation that reinforces repression of GA synthesis (an important action during seed maturation) but at the same time, and perhaps partially as a consequence of DELLA antagonism, promotes events important to subsequent germination.

METHODS

Plant Materials, Growth Conditions, and Generation of Transgenic Plants

Growth of Arabidopsis thaliana ecotype Columbia occurred in a growth chamber with a 16-h-light/8-h-dark cycle at 22°C under 150 to 175 μmol/m²/s light intensity for production of transgenic plants and seed collection. Transformation of Arabidopsis using the Agrobacterium tumefaciens strain GV3101:pMMP90 followed Clough and Bent (1998). Single-gene-copy homologous transgenic seed was carried through to the T2, T3, or T4 generation for subsequent phenotypic analyses. When comparing Columbia wild-type seed to ENY transgenic lines, seed was always grown at the same time and in the same growth chamber to ensure the most direct comparison was made. For overexpression of ENY, the ENY ORF was PCR amplified from seed cDNA and recombined into pDONR221 (Life Technologies). Next, the ENY ORF was recombined into the 2x35S expression vector pMDC32 (Curtis and Grossniklaus, 2003). The RAB18 promoter was also used to drive expression of ENY or an amiRNA designed for ENY. A 659-bp fragment of the RAB18 promoter was amplified from Columbia genomic DNA with SbfI and KpnI restriction sites and cloned into pCR2.1-TOPO (Life Technologies) before restriction digestion and ligation into pMDC32 to replace the 2x35S promoter. For GUS expression, a 2946-bp fragment of the upstream region of ENY was cloned in pDONR221 before being recombined into pMDC162 (Curtis and Grossniklaus, 2003). An amiRNA based on the mir319a backbone was designed using the MicroRNA Designer 2 (Schwab et al., 2006) and cloned into pDONR221 before cloning into the expression vectors pMDC32 and pMDC32-RAB18. Please refer to Supplemental Table 2 online for a list of PCR primers.

Seed Fresh/Dry Weight Analysis during Development

Flowers from ~90 to 100 plants of Columbia wild type and ENY misexpression lines were labeled at the time of anthesis with colored tape. Approximately 20 seeds were dissected from two to three silique and collected in prebaked and preweighed screw-cap HPLC glass vials (Agilent). For weighing, a scale capable of stably measuring 10⁻⁵ g was used. Following fresh weight measurement, seeds were baked for 24 h at 110°C and allowed to cool to room temperature in a desiccator before dry weight was measured.

Seed Mucilage Staining

To stain the acidic polysaccharides in Arabidopsis mucilage, mature seed of ENY misexpression lines were stained in 0.03% ruthenium red (Sigma-Aldrich) for ~2 to 3 min. Seeds were then rinsed with water and mounted on microscope slides before pictures were captured using a Zeiss SteREO Lumar.v12 stereomicroscope equipped with a 3.3 MP Zeiss iCC3 digital camera. In addition to ruthenium red, the fluorescent polysaccharide binding calcicoflor white (American Cyanamid) was used to stain mature seeds. A stock solution of 20 mg/mL calcicoflor white in 40 mM NaOH was diluted 20-fold in water to make a working solution of 1 mg/mL, and seeds were stained for ~5 min before rinsing with water twice. Seed images were captured with the above mentioned microscope and a 4',6-diamidino-2-phenylindole filter set. For EDTA treatment of 2x35S:ENY seeds, the procedure followed that described by Arsovski et al. (2009).

Resin Embedding for Light Microscopy

RESIN embedding of Columbia wild type and ENY-ME lines followed Western et al. (2000) with modifications. Developing seeds at 6 to 10 DPA, enclosed within partially opened silique, were fixed with 3% paraformaldehyde (Sigma-Aldrich) and 1.25% glutaraldehyde (Sigma-Aldrich) in 0.5 M sodium phosphate buffer, pH 7.0, overnight at 4°C. Following brief rinses with phosphate buffer, the seeds were dehydrated in a graded ethanol series for 5 d. The seeds were then gradually infiltrated with LR White resin (Ted Pella) over a period of 5 d. LR White was polymerized at 57°C in gelatin capsules for 24 h. Seeds were sectioned at 1 μm on a Leica RM2165 microtome before mounting on Probe-On Plus slides (Fisher Scientific). Sections were stained briefly with 1% (w/v) toluidine blue O in 1% (w/v) sodium borate, pH 11.0, before sealing with VectaMount mounting medium (Vector Labs). Pictures were captured using a ×20 objective on a Leica DMR microscope and MacroFire CCD camera (Optronics).

GUS Staining during Seed Development

Siliques were labeled and seed was collected at 3, 6, 9, 12, 15, and 18 DPA and fixed in 20% acetonitrile for >24 h at ~20°C. The same procedure was followed for seeds collected after various stages of imbibition and germination. Seeds were opened to allow easier access to the GUS reagent. Following a brief vacuum infiltration, seeds were incubated in 0.1 M NaPO₄, pH 7, 10 mM EDTA, 0.1% Triton X-100, 1.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and 2.0 mM X-Gluc (Roes Scientific) for 30 min to 1 h at 37°C. Seeds were preserved in 70% ethanol before capturing images with the Leica and Zeiss microscopes detailed above.

Vertical Agar Plate Growth Assays

Following surface sterilization in 70% ethanol and 10% commercial bleach, and two rinses with sterile water, seeds were resuspended in 0.2% agar. Columbia wild type and 2x3SS:ENY T3 seeds were placed on 0.5 MS, 2 mM MES, pH 5.8, plus 1% agar with or without the addition of 1% Suc at a density of 12 to 15 seeds per plate (10 × 1.5-cm square plates; BD Falcon). Seeds were moist chilled for 5 d before plates were placed at 22°C for 7 d (16-h-light/8-h-dark cycle; 70 to 80 μmol/m²/s). A Canon Powershot G10 camera was used to capture images before measurement in ImageJ (Abramoff et al., 2004).

For ENY induction experiments with ABA, T2 transgenic lines of RAB18:ENY were plated at a density of ~20 seedlings per plate on 0.5 MS, 2 mM MES, pH 5.8 plus 1% agar. Following 3 d of moist chilling and 5 d under germination conditions (as above), seedlings were transferred to plates containing 10 μM (+)-ABA and left on the plates for 1 to 6 h. In a separate experiment, used for qRT-PCR expression analysis, seedlings were collected following a 3-h treatment on plates containing ABA with or without the inclusion of 10 μM GA₃ or 1 μM Paclobutrazol (Sigma-Aldrich).

RNA Extraction

The following tissue amounts from Arabidopsis were generally used for RNA extraction: 50 to 100 siliques containing seeds for time points during seed development, 40 to 50 mg fresh weight of dry mature seed (including those time points that were extracted following imbibition), or 24 to 32 5-d-old seedlings. All tissues were frozen in liquid N₂ prior to grinding. For dry and imbibed seeds, frozen tissue was equilibrated with RNAwater-ICE.
Gene Expression Analysis

For cDNA synthesis, 1 μg of RNA was used with the Superscript III First-Strand Synthesis Supermix for qRT-PCR (Life Technologies) according to the manufacturer’s instructions except that the cDNA synthesis reaction was increased to 60 min. cDNA was diluted 12.5-fold in water, and 2 to 8 μL of this dilution was used for qRT-PCR expression analysis. For qPCR, SYBR GreenER qPCR Supermix Universal (Life Technologies) was used in a total reaction volume of 25 μL, with 0.1 μL ROX reference dye and 0.2 μM primer concentration. Data were collected as Ct values in an MX3000P instrument with Plant RNA Isolation Aid according to the manufacturer’s protocol (Life Technologies). Following RNA extraction, RNA was treated with DNase using the TURBO DNA-free kit (Life Technologies). RNA quality was analyzed with an RNA Nano chip using the Bioanalyzer 2100 (Agilent) prior to cDNA synthesis.

Microarray Analysis of ENY Transgenic Lines

RNA samples for microarray analysis were extracted from two biological replicates that were set up and collected independently. Thus, each microarray time point consisted of two biological replicates and two to three technical replicates, which included dye-swaps (thus, four slides per time point was the minimum). Two color array experiments were performed using arrays based on the Operon Arabidopsis Genome Array Ready Oligo Set (AROS) Version 3.0 (http://omad.operon.com/download/index.php) and were obtained from D.W. Galbraith’s Lab at the University of Arizona (http://ag.arizona.edu/microarray). Two color microarray experiments were always a transgenic ENY line compared with Columbia wild type.

For qPCR gene expression measurements during seed development, 900 ng of RNA was used with the SuperScript VILO cDNA synthesis kit with a synthesis reaction of 65 min. cDNA was diluted 14-fold in water, and 5 μL of this dilution was used in each qPCR reaction. qPCR was performed as detailed above. To obtain copy numbers for gene expression, the formula mass (g) = DNA (bp) * 1 mol/6.023 x 10^{23} molecules (bp) * 660 g/mole was used to equate the plasmid mass with DNA base pair size (660 was used as the average weight for a double-stranded DNA molecule). Linearized plasmid templates, including the ENY, RGA, GAI, RGL1, RGL3, and SCL3 ORF or RGL2 cDNA, were used as templates and standard curves varying from 300,000 to 30 copies were generated. Standard curves relating copy number to CT values were generated alongside the experimental samples. UBC21 was selected as a reference gene based on Czechowski et al. (2005).

Comparisons to Red Light, Quadruple pif, and ga1-3 Microarray Data Sets

Microarray data comparing (1) Col-0 seed treated with either an FR light pulse or FR/R light pulses and imbibed for 12 in the dark (Col-0(R) vs Col-0 (D)) (Okamoto et al., 2009), (2) Col-0 seed compared with pifq seed after 5 d of moist chilling [pifq(seed) versus Col-0(seed)] (Leivar et al., 2009), and (3) ga1-3 seed treated with 5 μM GA_{3} to ga1-3 treated with water after 9 h white light imbibition [ga1-3(GA)/ga1-3(water)] (Ogawa et al., 2003; Goda et al., 2008) were downloaded from the GEO (Barrett et al., 2009) as Affymetrix CEL files (GEO accesses GSE14374, GSE17159, and GSE5701, respectively). CEL files were inputted into GeneSpring GX10 using the GCRMA algorithm and performing the Baseline to Median of All Samples adjustment. Following Oh et al. (2009), probes with low signal intensities (<64) were filtered out of the analysis. Normalized log2 expression values were used for SAM. SAM gene lists were imported back into GeneSpring to identify DEGs with a fold change ≥1.5. DEGs from RAB18:ENY1-h imbibed seed arrays were imported into GeneSpring and compared with the DEGs in the Col-0(D) versus Col-0(R) and Col-0(seed) versus pifq(seed) data sets. For comparison of RAB18:ENY and RAB18:ENYami DEGs to red light and ga1-3 microarray data, low-signal-filtered data were exported into a new GeneSpring experiment and expressed as a log2 ratio [i.e., ratios of Col-0(R)/Col-0(D) and ga1-3(GA)/ga1-3(water)]. RAB18:ENY and RAB18:ENYami DEGs were imported and compared using a scatterplot in GeneSpring. Final data were plotted in SigmaPlot 9 (Systat Software).

Generally, 1 μg of RNA was used with the Amino Allyl MessageAmp II aRNA amplification kit (Applied Biosystems) to produce amplified antisense RNA (aRNA). Five micrograms of Alexa 555 and 647 (Life Technologies) labeled aRNA was hybridized to Arabidopsis 70mer oligonucleotide microarray slides. A microarray slide covered with a LifterSlip (Erie Scientific) was placed into a hybridization chamber (Arrayit). The hybridization solution consisted of 70 μL of SlideHyb1 (Applied Biosystems) and ~5 μL of each Alexa-labeled fragmented aRNA pool. Following hybridization at 45°C for 16 to 18 h, slides were washed before spin drying. Slide scanning was accomplished using an Axon 4000B scanner (Molecular Devices) using the 532- and 635-nm laser channels and a resolution of 10 μm/pixel. Data (median foreground – median background) were imported into GeneSpring GX10 for analysis using Sub-Grid LOWESS normalization (Smyth and Speed, 2003), and spots representing low signal intensity (<80) were filtered out of the analysis.

Gene annotations were updated for TAIR9 and BIN annotations were added from the MapMan Site of Analysis (Thimm et al., 2004). Normalized log2 values were used to identify significant genes using a modified t test with SAM in Microsoft Excel (Tusher et al., 2001). An FDR of 0.05 (q-value of 5) was used as a cutoff for SAM (i.e., five genes out of 100 could be expected to be incorrectly associated as significantly changed). SAM gene lists were imported back into GeneSpring to identify DEGs with a fold change ≥1.5. Additional analyses of DEG lists included the use of Athena (O’Connor et al., 2005) to identify enriched promoter elements within 1000 bp and P values < 10^{-6} and GO terms with P values < 10^{-3}.

For RAB18:ENYami microarray analysis, Agilent Technologies Arabidopsis 4x44k arrays (version 4) and Low Input Quick Amp Labeling Kit (Agilent Technologies) were used. The protocol followed that detailed by the manufacturer. Two hundred nanograms of total RNA was chosen as starting material for subsequent cDNA production, and the cRNA amplification reaction proceeded for 2.5 h. Two micrograms each of cyanine 3- and 5-labeled amplified cRNA was hybridized to each array. Following washing with Agilent stabilization and drying solution, arrays were scanned individually using an Axon 4000B scanner with a resolution of 5 μm/pixel. Data analysis followed the same protocol detailed above.
Extraction and Quantification of ABA

Following lyophilization for 3 d, seeds were extracted and ABA and dihydrophaseic acid were quantitated by the method described by Kong et al. (2008). The hormone profiling analysis was performed by HPLC-electrospray tandem mass spectrometry using deuterated internal standards by the hormone profiling service at the Plant Biotechnology Institute.

Yeast Two-Hybrid Analysis

Protein–protein interaction analysis was performed using the Matchmaker Gold yeast two-hybrid system (Clontech). The ORF of ENY was cloned into bait vectors pGBK7 and pGBT9 using the In-Fusion Advantage PCR cloning kit (Clontech). Testing for autoactivation and toxicity of ENY followed the manufacturer’s instructions. ENY-pGBK7, previously transformed into the Y2H Gold yeast strain, was used to screen a commercially available Mate and Plate Library (Clontech). This library was constructed from normalized Arabidopsis cdNA from several tissue sources, including pollen, open and closed flowers, seedlings, etiolated seedlings, siliques with seeds (3 to ~10 DPA), leaves before and after bolting, and stems. Following a 24-h mating, library screening was performed on SD/-Leu/-Trp/-His/-Ade medium with 78 ng/mL Aureobasidin A (Clontech) and 20 μg/mL x-α-Gal (Gold Biotechnology) (QDO/X/A). Following 4 d of growth at 30°C, blue colonies with appreciable growth were streaked onto fresh QDO/X/A and grown for a further 3 d. Yeast plasmids were extracted using the Easy Yeast Plasmid Isolation Kit (Clontech), and cDNA inserts were PCR amplified and sequenced from Yeast plasmids.

For in vivo staining of pollen tubes, pistils from Stage 12-14 flowers (Smyth et al., 1990) were fixed overnight in 90% ethanol and 1% acetic acid. Following rinsing in a graded ethanol series (to 50%) for 20 min each, seeds were allowed to clear in Hoyer’s solution for 2 h. Pistils were then treated with 1 M NaOH for 3 h before staining with decolorized aniline blue overnight. Pistils were then briefly rinsed with 0.1 M K2HPO4 (pH 11 with KOH) and mounted on a microscope slide with 33% glycerol and 0.1 M K2HPO4. Images were captured using a Leica DFC320 digital camera on a Leitz Orthoplan microscope with a 4',6-diamidino-2-phenylindole filter set.

Full-length siliques were removed from three to five plants of Columbia wild type and 2x3SS:ENY lines. Siliques were arranged and numbered on a white piece of paper with a centimeter ruler, and photographs were taken with a tripod-mounted Canon Powershot G10 digital camera. Silique length was measured using ImageJ. Each plant line had at least 60 individual silique measurements. Following capture with the Powershot G10, siliques were dissected and seeds within each silique were counted. Data were compiled in Microsoft Excel and plotted using SigmaPlot 9.

Oil Analysis

The fatty acid profiles of 10 mg seed from Columbia wild type and 2x3SS:ENY lines were determined by gas chromatography using 17:0 fatty acid methyl ester as an internal standard as described by Taylor et al. (1991), Katavic et al. (1995), and Zheng et al. (2003). Data were compiled in Microsoft Excel and plotted using SigmaPlot 9.

Protein Measurement

For total protein measurements, seed was harvested from two independently grown groups of ~90 plants, and seed protein was measured with two technical replicates. Proteins were extracted from 25 mg mature seed of Columbia wild type and 2x3SS:ENY lines with 1 mL of 50 mM Tris-HCl, pH 8.8, 6 M Urea, 1% SDS, and 5 mM DTT. Samples were ground with ceramic spheres (Q-Biogene) in 2-mL screw cap microcentrifuge tubes with a Fast Prep FP120 machine (Q-Biogene). Following a 25-min incubation at room temperature, samples were spun at 16,000 g for 10 min at 4°C. The pellet was reextracted sequentially with 0.5 and 0.25 mL of extraction buffer. Protein in the combined supernatants was quantitated using the RC-DC protein assay (Bio-Rad) using 2 mg/mL BSA (Bio-Rad) as a standard. Data were compiled in Microsoft Excel and plotted using SigmaPlot 9.

Sugar Analyses

The method for sugar extraction followed that described by Bock et al. (2009) but was modified for extraction from Arabidopsis and for starch. Pyrex (16 mm) tubes were rinsed twice with dichloromethane, air dried, and baked to remove any oil residue. One hundred milligrams of mature Arabidopsis seed from Columbia wild type and 2x3SS:ENY lines was ground in 2 mL 2:1 dichloromethane/isopropanol with an Omni Mixer Homogenizer (Omni International). The samples were spun at 1250 g for 5 min, and the supernatant was removed and the pellet rinsed again with 2:1 dichloromethane/isopropanol. The pellet was dried under a gentle nitrogen stream for 2 to 3 min. The pellet was reresuspended in 1 mL 80%
ethanol for 30 min with periodic vortexing (lactose was added at this step as a recovery standard). The samples were spun at 1250g, and the supernatant was removed to 2-mL microcentrifuge tubes. The pellet was resuspended in 250 μL water by vortexing for 10 min. The samples were filtered through a 0.45-μm hydrophilic polypropylene membrane (GH Polypro Acrodisc; Pall Life Sciences) before adding to a HPLC vial.

The pellet from above was used to extract starch. The pellet was baked at 50°C for 30 min before adding 200 μL 0.5 M NaOH and 200 μL water and baking at 70°C for 1 h. The reaction was equilibrated with 200 μL 0.5 M HCl allowed to cool, and 400 μL 0.2 M NaOAc, pH 4.8, was added. Two units, dissolved in 0.2 M NaOAc, of amyloglucosidase (Fluka) was added to the 1-mL suspension and mixed gently. After overnight digestion at 37°C, the reaction was stopped by heating for 10 min at 100°C. After spinning for 5 min at 1250g, the supernatant was transferred to a 2-mL tube, spun for 3 min at 20,000 g, and filtered through a 0.45-μm hydrophilic polypropylene membrane (GH Polypro Acrodisc; Pall Life Sciences) before adding 250 μL to an HPLC vial.

The sugar analysis with a Dionex Bio-LC system was performed as described by Bock et al. (2009). Data were compiled in Microsoft Excel and plotted using SigmaPlot 9.

Germination Assays

Following seed sterilization, triplicates of 50 seeds were plated in 9-cm Petri dishes with two layers of 7-cm Whatman #1 Filter Paper (Filter Scientific) with 2 mL of water and left under fluorescent white light for 1 h. Seeds were treated with 4 μmol/m2/s FR light for 6 min and 4 μmol/m2/s FR light for 6 min and 30 μmol/m2/s R light for 10 min using a SNAP-LITE lighting system with 670- and 735-nm LED arrays (Quantum Devices). Seed germination was scored after 4 d in the dark. A seed was deemed germinated if the radicle had visibly protruded through the seed coat.

Germination on exogenous ABA followed Cutler et al. (2000).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ID11/ENY (AT5G66730), AB1 (AT4G26080), ACT2 (AT3G18780), AGF1 (AT1G35390), ALC (AT5G67110), ABTH2 (AT4G16780), BAS1 (AT2G26710), BRRX2 (AT3G30180), COLD1 (AT3G13550), DOGT1 (AT2G36800), EXPAT1 (AT1G69530), FUS3 (AT3G26790), GA2 (AT1G17060), GA3 (AT5G29590), GA20X1 (AT1G78440), GA20X2 (AT5G1810), GA20X3/YAP169 (AT5G02700), GA20X6 (AT1G02400), GA3OX1/GA4 (AT1G15550), GA3OX2/GA4H (AT1G80340), GAI (AT1G4920), GID1A (AT3G05120), GIB1B (AT3G63010), GNC (AT5G68860), ID3/MAG (AT1G30490), ID8/NUC (AT5G4169), ID10/JO (AT5G03150), ID15/SAG5 (AT2G01940), JA21 (AT1G19180), LAF3 (AT3G55850), PLY11 (AT5G45860), PHYA (AT1G09570), RAB18 (AT5G66400), RGA (AT2G01570), RGL1 (AT1G66350), RGL2 (AT3G03450), RGL3 (AT5G17490), SCL3 (AT1G50420), SCR (AT3G4220), SHR (AT4G37650), SLY1 (AT4G2420), SOB7 (AT1G17060), SPA3 (AT3G15354), UBC21/PEX4 (AT5G25760), UBO11 (AT4G05050), tobacco RSG (BAA97100), and rice YABBY1 (AF098752).

Supplemental Data

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

Supplemental Figure 1. Defective Muclilage Extrusion in ENY Misexpressing Lines.

Supplemental Figure 2. Cation Chelation Promotes Muclilage Release in ENY Misexpressing Lines.

Supplemental Figure 3. Anther Dehiscence Is Delayed and Inhibited in ENY-ME Lines.

Supplemental Figure 4. Seed Production Is Lower in ENY-ME Lines Due to the Polllination Defect.

Supplemental Figure 5. Seeds in Siliques during Development of ENY-ME lines and Columbia Wild Type.

Supplemental Figure 6. Mature Embryos of ENY-ME Lines Were Morphologically Similar to Columbia Wild Type but Were More Translucent in Appearance.

Supplemental Figure 7. Storage Product Synthesis and Partitioning Are Disrupted in ENY-ME Lines.

Supplemental Figure 8. Muclilage Extrusion in RAB18:ENY Transgenic Lines Is Similar to Columbia Wild Type.

Supplemental Figure 9. Induction of ENY Expression in ENY Transgenic Lines Driven by the RAB18 Promoter.

Supplemental Figure 10. Validation of Microarray Results.

Supplemental Figure 11. Growth of ENY Misexpression Seedlings under Gibberellin Treatment Results in a Skotomorphogenic-Like Phenotype.

Supplemental Figure 12. Hypocotyl Lengths of RAB18:ENY Lines 2 and 13 d Seedlings under ABA and GA Treatment.

Supplemental Figure 13. Expression of ENY in RAB18 Overexpressing and Knockdown Lines.

Supplemental Figure 14. ENY Promotes Germination during Far-Red and Red Light Treatments.

Supplemental Figure 15. Germination of ENY Misexpressing Lines with and without Various Durations of Moist Chilling.

Supplemental Figure 16. ABA Sensitivity of ENY Transgenic Lines during Germination.

Supplemental Figure 17. Interaction of ENY and DELLA Proteins.

Supplemental Figure 18. Nuclear Localization of YFP-ENY.

Supplemental Table 1. Expression of Genes Associated with Hormonal or Light Signaling in Imibed Seeds of RAB18:ENY and RAB18:ENYam Transgenic Lines.

Supplemental Table 2. List of PCR Primers Used in This Study.

Supplemental Data Set 1. Differentially Expressed Genes from 5 and 10 Days Postanthesis and Mature Seeds of ENY-ME Seed Compared with Columbia Wild Type.

Supplemental Data Set 2. Differentially Expressed Genes from RAB18:ENY Induction Experiment with Abscisic Acid.


Supplemental Data Set 4. Differentially Expressed Genes from RAB18:ENYami 1 h Imibed Seeds.

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The *Arabidopsis* C2H2 Zinc Finger INDETERMINATE DOMAIN1/ENHYDROUS Promotes the Transition to Germination by Regulating Light and Hormonal Signaling during Seed Maturation

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