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Far-red light inhibits germination through DELLA-dependent stimulation of ABA synthesis and ABI3 activity

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Under the canopy, far-red (FR) light represses seed germination by inactivating phytochrome photoreceptors. This elicits a decrease in gibberellins (GA) levels and an increase in abscisic acid (ABA) levels. GA promotes germination by enhancing the proteasome-mediated destruction of DELLA repressors. ABA prevents germination by stimulating the expression of ABI repressors. How phytochromes elicit changes in hormone levels or how GA- and ABA-dependent signals are coordinated to repress germination remains poorly understood. We show that repression of germination by FR light involves stabilized DELLA factors GAI, RGA and RGL2 that stimulate endogenous ABA synthesis. In turn, ABA blocks germination through the transcription factor ABI3. The role of PIL5, a basic helix-loop-helix transcription factor stimulating *GAI* and *RGA* expression, is significant, provided GA synthesis is high enough; otherwise, high GAI and RGA protein levels persist to block germination. Under white light, *GAI* and *RGA* driven by the *RGL2* promoter can substitute for *RGL2* to promote ABA synthesis and repress germination, consistent with the recent findings with *RGL2*. The three DELLA factors inhibit testa rupture whereas ABI3 blocks endosperm rupture.

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Introduction

In Arabidopsis, the mature seed consists of a protective outer layer of dead tissue, the testa, under which the endosperm, a single layer of cells, surrounds the embryo (Debeaujon *et al*, 2000). Arabidopsis seed germination chronologically involves testa rupture followed by concomitant endosperm rupture and embryonic axis (i.e. radicle) protrusion, the latter being the usual definition of germination (Kucera *et al*, 2005; Muller *et al*, 2006). When seeds are non-dormant, as in this study, imbibition by water is sufficient to trigger germination. Under normal conditions, the process can be completed within 48–72 h after seed imbibition (Piskurewicz *et al*, 2008).

Germination is under tight control by the environment, being affected by light quality, temperature and water potential. Environmental factors eventually determine the relative levels of the phytohormones gibberellins (GA) and abscisic acid (ABA) that have an antagonistic and important function in the control of seed germination (Olszewski *et al*, 2002; Nambara and Marion-Poll, 2005). Conditions favourable for germination lead shortly after seed imbibition to an increase of GA levels (Ogawa *et al*, 2003), which is essential for germination to occur. In parallel, the levels of ABA drop rapidly after dry seed imbibition, and thereafter the role of ABA becomes facultative: a sudden osmotic stress or direct ABA application effectively blocks endosperm rupture and its effect on testa rupture is significantly lesser (Muller *et al*, 2006; Piskurewicz *et al*, 2008). Importantly, exogenous GA does not stimulate endosperm rupture in ABA-treated seeds, suggesting that ABA acts downstream of GA for the control of endosperm rupture (Muller *et al*, 2006). The ABA-dependent growth arrest occurs only within a limited time window of about 48 h after seed imbibition (Lopez-Molina *et al*, 2001). ABA or osmotic stress stimulates the *de novo* accumulation of embryonic transcription factors ABI3 and ABI5, which are both necessary to repress germination (Lopez-Molina *et al*, 2001, 2002). Similarly, as during seed maturation, ABI5 expression is positively regulated by ABI3 and both transcription factors ensure the *de novo* induction of *LEA* gene expression, thus maintaining the embryonic nature of the arrested embryo (Parcy *et al*, 1994; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000).

GA promotes seed germination by enhancing the destruction of the DELLA repressor proteins through the 26S-proteasome machinery. DELLA genes are predicted to encode GRAS-family transcription factors, although no DNA-binding activity has been characterized so far. Rather, it has been proposed that they influence transcription by interacting with other DNA-binding transcription factors (Zentella *et al*, 2007). In the proposed model, GA binds to Arabidopsis GID1-like receptors, so as to enhance DELLA protein interaction with the F-box protein SLY1, thus facilitating DELLA ubiquitination and subsequent degradation (Sun and Gubler, 2004; Feng *et al*, 2008). There are five DELLA genes in the Arabidopsis genome: *RGA*, *GAI*, *RGL1*, *RGL2* and *RGL3*. Even though all DELLA genes are expressed during seed germination, only *RGL2*, *GAI* and *RGA* have been shown to have a function to repress germination (Lee *et al*, 2002; Tyler *et al*, 2004; Penfield *et al*, 2006).

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Light quality affects seed germination by invoking changes in GA and ABA levels (Seo *et al*, 2006). This notably involves phytochrome B (phyB) and PIL5, a basic helix-loop-helix (bHLH) transcription factor indirectly modulating GA and ABA levels and directly stimulating *RGA* and *GAI* gene transcription (Bae and Choi, 2008; Seo *et al*, 2009). phyB is a protein photoreceptor with a covalently attached light-sensitive chromophore, whose activity is mainly set by the intensity ratio of red light to far-red (FR) light. An elevated ratio of red light to FR light (R conditions) leads to the active state of phyB (P_{fr} state). When active, phyB triggers the proteasome-mediated destruction of PIL5 through a process in which phyB and PIL5 interact (Oh *et al*, 2004, 2006). As a result, GA synthesis occurs normally and *GAI* and *RGA* transcription is low (Oh *et al*, 2007).

In contrast, under the canopy, a low ratio of red to FR light (FR conditions) will inactivate phyB (P_r state) (Chen *et al*, 2004). When inactive, phyB no longer interacts with PIL5 and this prevents PIL5 destruction by the 26S-proteasome machinery. As a result, PIL5 accumulates and represses seed germination. Under this model, PIL5-dependent repression involves (1) GAI and RGA, (2) SOMNUS (*SOM*), a CCCH-type zinc-finger protein also repressing germination and (3) regulation of ABA and GA metabolic genes (Kim *et al*, 2008; Seo *et al*, 2009). PIL5 activates the transcription of *GAI, RGA* and *SOM* genes by directly binding to their promoter sequences. In parallel, both PIL5 and SOMNUS are proposed to indirectly lower GA levels and elevate ABA levels by modulating the expression of GA and ABA metabolic genes in an unknown manner. In turn, lower GA levels further increase DELLA protein stability, thus enhancing RGA-, GAI- and RGL2-dependent repression, whereas higher ABA levels repress seed germination by stimulating *ABI3* and *ABI5* expression.

Some key aspects of the model described above have not been addressed. First, *ga1/pil5* (and *ga1/som*) double mutants cannot germinate under FR conditions, suggesting that additional unidentified repressive activities occur when GA synthesis is severely prevented (by the *ga1* mutation). Second, the role of ABA and ABA-response factors to repress germination has not been directly investigated. Third, the nature of the PIL5- and SOM-dependent regulation of the expression of genes involved in hormone metabolism is unclear (Oh *et al*, 2007; Kim *et al*, 2008). Indeed, the observed changes in the expression of genes involved in GA and ABA metabolism in *pil5* or *som* mutants may be interpreted as secondary effects resulting from *pil5* and *som* seed germination under FR conditions. Finally, the notion that the balance of GA and ABA levels determines the germination potential of the Arabidopsis seed lacks precision in terms of their effects on development. Thus, even though the observation that GA and ABA levels are inversely correlated strongly points to a regulatory crosstalk between their metabolic pathways, this does not necessarily imply that each hormone regulates germination through symmetrically inversed mechanisms. Indeed, in a recent report, we showed that RGL2 and ABI5 have distinct developmental functions under white light that are consistent with how GA and ABA differently influence testa and endosperm rupture. In response to low GA levels, a stabilized RGL2 efficiently blocks testa rupture and increases endogenous ABA levels. ABA in turn stimulates *ABI5* expression and product activity to repress endosperm rupture (Piskurewicz *et al*, 2008). This points to the general notion that GA promotes testa rupture and limits ABA levels, thus ensuring low expression and activity of the ABA-response factors repressing endosperm rupture.

Here, we report that this view can be extended to the phyB-dependent control of seed germination. We show that under FR conditions, low GA levels allow an overaccumulation of GAI, RGA and RGL2, which block testa rupture and promote an increase in the levels of ABA, ultimately responsible to prevent endosperm rupture. This may involve DELLA-dependent stimulation of the expression of *XERICO*, encoding an RING-H2 factor promoting ABA synthesis in an unknown manner. ABA-dependent repression under FR conditions is orchestrated by ABI3. We show that the role of PIL5 to influence GAI and RGA protein levels is limited to FR conditions in which GA levels are not drastically reduced. As a result, in *pil5* seeds under low GA conditions, GAI and RGA levels increase sufficiently to block testa rupture and promote higher endogenous ABA levels. Finally, we show that *GAI* and *RGA* can complement *rgl2* mutants when both are under the control of *RGL2* promoter sequences, further confirming their role to repress testa rupture and to stimulate endogenous ABA synthesis during germination.

Results

RGL2, GAI and RGA are the main GA-response repressors of testa and endosperm rupture under FR light

Under phyB-dependent repression of seed germination, a pulse of FR light (FR conditions) ensures a complete blockade of seed germination in darkness unless followed by a pulse of red light (R conditions, phyB is active). FR conditions (i.e. phyB is inactive) are associated with lower GA levels and higher ABA levels, whereas the converse (high GA, low ABA) occurs under R conditions (Seo *et al*, 2006). The relative role of each hormone as well as each GA- or ABA-response factor to repress seed germination remains unclear.

Earlier reports have suggested that in conditions other than white light illumination, RGL2 is no longer the main DELLA factor repressing seed germination in response to low GA levels (Cao *et al*, 2005). Indeed, contrarily to what is observed under white light conditions, *ga1/rgl2* double mutants can no longer germinate in darkness. In contrast, *ga1/rgl2/gai/rga* mutants can germinate in the dark, which shows that RGL2, GAI and RGA redundantly repress germination in the absence of light. However, darkness is a rather ill-defined treatment from the perspective of phytochrome-dependent repression of seed germination. Indeed, varied seed germination responses may occur in darkness depending on the seed batch, including germination of the entire seed population (Cao *et al*, 2005; Supplementary Figure 1). This is most likely because of the presence of different pools of active phyB in imbibed seeds that could be inherited from embryogenesis or from the experimental manipulations performed in the presence of light before seed imbibition. Therefore, seed germination in *ga1/rgl2/gai/rga* could still involve active phyB, so that the relative contribution of phyB and DELLA factors remains unclear.

Here, we explore the role of RGL2, GAI and RGA together with ABA to repress seed germination under conditions in which phyB is inactive (i.e. FR conditions). A 5-min FR

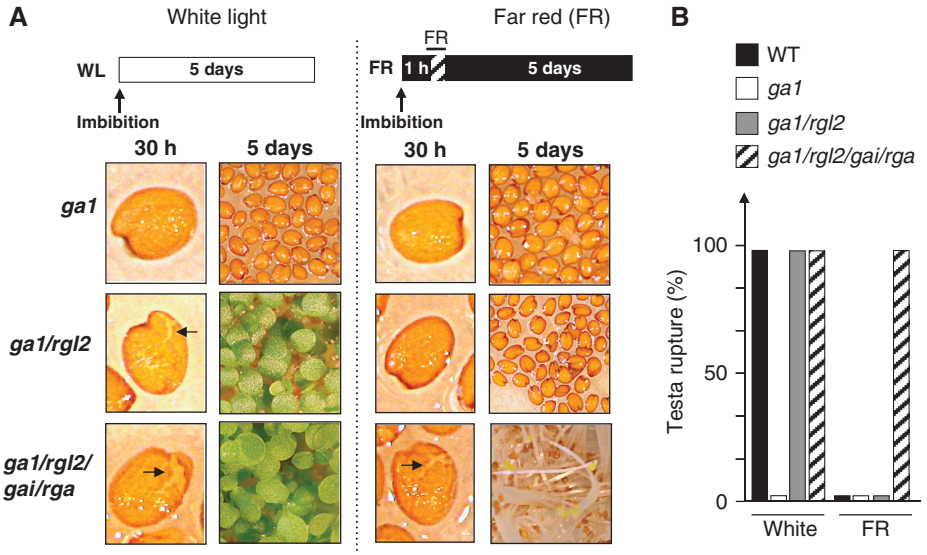

Figure 1 RGL2, GAI and RGA redundantly repress testa rupture under FR conditions. (**A**) Representative pictures showing *ga1-3*, *ga1-3/rgl2-13* and *ga1-3/rga-t2/gai-t6/rgl2-1* seeds 30 h and 5 days after imbibition under white light conditions or far-red (FR) light conditions. Picture showing testa rupture in *ga1-3/rgl2-13* seed is taken 72 h after imbibition. Arrows indicate testa rupture events. (**B**) Histogram shows percentage of testa rupture events 5 days after imbibition of WT (Col), *ga1-3*, *ga1-3/rgl2-13* and *ga1-3/rgat2/gait6/rgl2-1* seeds under white (white) or FR light conditions (in three independent seed batches ($n = 150$–300) testa rupture percentage is always either 0 or 100% depending on the genotype and the light conditions as shown in the histogram).

irradiation treatment is sufficient to inactivate phyB and ensure repression of germination in darkness (Supplementary Figure 1). We first examined testa and endosperm rupture events in *ga1*, *ga1/rgl2* and *ga1/rgl2/gai/rga* seeds under FR conditions, a situation that had not been yet explored.

Under white light conditions, testa and endosperm rupture could be observed in *ga1/rgl2* and *ga1/rgl2/gai/rga* seeds, but not in *ga1* seeds, consistent with the earlier results (Figure 1A and B). In contrast, under FR conditions, testa rupture and endosperm rupture could only be observed in *ga1/rgl2/gai/rga* seeds (Figure 1A and B). FR conditions were associated with higher GAI and RGA protein contents in *ga1* and *ga1/rgl2*, consistent with the earlier reports (Supplementary Figure 2). This further supports the notion that GAI and RGA, together with RGL2, become key GA-response repressors of testa and endosperm rupture when the phytochrome is inactive.

GAI, RGA and RGL2 are necessary to increase endogenous ABA levels under FR conditions

Earlier reports have suggested that GAI and RGA may stimulate endogenous ABA synthesis in seedlings (Zentella *et al*, 2007). In addition, we showed earlier that RGL2-dependent repression of seed germination in white light involves stimulation of endogenous ABA synthesis (Piskurewicz *et al*, 2008). We thus hypothesized that under FR conditions, GAI and RGA, together with RGL2, are necessary to repress germination also by stimulating ABA synthesis.

To address this possibility, we monitored endogenous ABA levels as well as ABI5 accumulation in protein blots, as it is a convenient marker for changes in endogenous ABA levels on seed imbibition and before seedling establishment (Piskurewicz *et al*, 2008). ABA levels, high in dry seeds, dropped markedly after imbibition under white light conditions in all *ga1*, *ga1/rgl2* and *ga1/rgl2/gai/rga* seeds, consistent with the earlier results (Figure 2A) (Piskurewicz *et al*, 2008). Thereafter, ABA levels remained 5–10-fold higher in *ga1* relative to *ga1/rgl2* or *ga1/rgl2/gai/rga* seeds, consistent with the earlier results (Figure 2A) (Piskurewicz *et al*, 2008). As expected, ABI5 protein levels were high in *ga1* seeds, but rapidly decayed in *ga1/rgl2* or *ga1/rgl2/gai/rga* seeds under white light conditions (Figure 2C). In contrast, endogenous ABA and ABI5 protein levels remained elevated in *ga1* and *ga1/rgl2* seeds under FR conditions (Figure 2B and C). In contrast, *ga1/rgl2/gai/rga* seeds maintained 5–10-fold lower endogenous ABA levels over time relative to *ga1* and *ga1/rgl2* and failed to maintain ABI5 protein levels (Figure 2B and C). Higher endogenous ABA levels were consistently associated with higher mRNA levels of *NCED6*, encoding a 9-cis-epoxycarotenoid dioxygenase involved in the first-step specific to ABA biosynthesis (Supplementary Figure 3) (Lefebvre *et al*, 2006). This is consistent with their ability to germinate and indicates that GAI and RGA, together with RGL2, are necessary to maintain high endogenous ABA levels under FR conditions.

XERICO encodes a putative RING-H2 factor promoting ABA synthesis in an unknown manner (Ko *et al*, 2006; Zentella *et al*, 2007). We showed earlier that under white light conditions, *RGL2* is necessary to elevate *XERICO* mRNA expression under low GA conditions (Piskurewicz *et al*, 2008). This suggested a possible mechanism accounting for the elevation of endogenous ABA levels after seed imbibition under low GA conditions. A similar mechanism, involving *GAI* and *RGA*, had been proposed earlier in seedlings, in which GAI and RGA bind *XERICO* promoter sequences and positively regulate its mRNA accumulation (Ko *et al*, 2006; Zentella *et al*, 2007). We, therefore, wished to explore whether GAI and RGA are necessary to promote *XERICO* mRNA accumulation under white and FR light conditions during seed germination and under low GA conditions. Supplementary Figure 4 shows that under white light conditions, *XERICO* mRNA expression

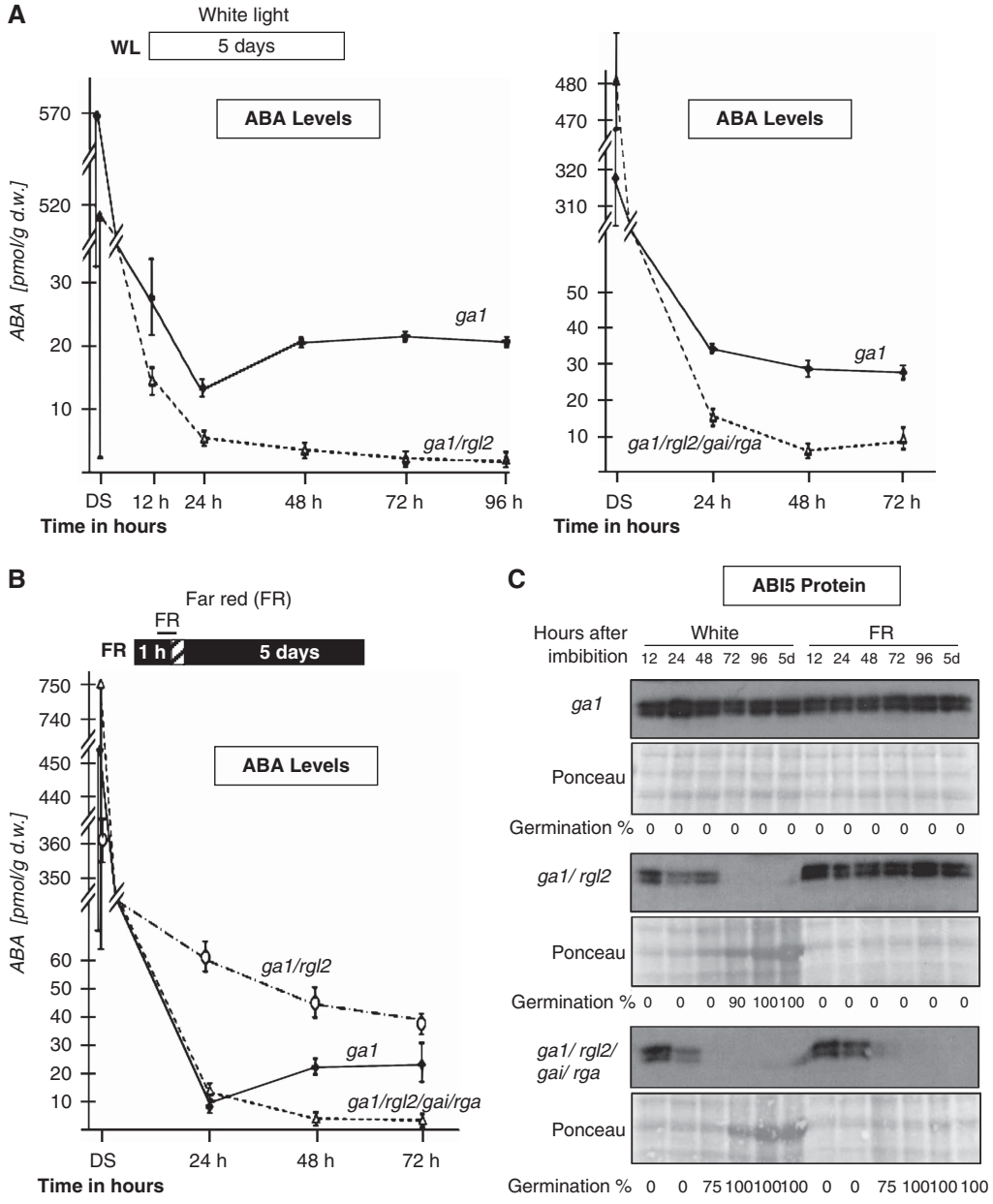

Figure 2 RGL2, GAI and RGA are necessary to promote endogenous ABA and ABI5 levels under FR conditions. (**A**) Time course of endogenous ABA levels in *ga1-3*, *ga1-3/rgl2-13* and *ga1-3/rga-t2/gai-t6/rgl2-1* seeds under white light conditions (normal MS medium). Units are pmol per gram of fresh weight. Error bars indicate s.d. ($n = 3$). (**B**) Same as in (A) under far-red light conditions. (**C**) Protein gel blot analysis of a time course of ABI5 protein levels upon *ga1-3*, *ga1-3/rgl2-13* and *ga1-3/rga-t2/gai-t6/rgl2-1* seed imbibition under far-red (FR) or white (white) light conditions. Signals can be directly compared between different genetic backgrounds. Each lane contains proteins extracted from 5 mg of seeds stained with Ponceau S (Ponceau) before incubation with antibodies against ABI5. At each time point, the percentage of germination (i.e. endosperm rupture events) in the seed population material used in the protein blot is indicated. We measured endosperm rupture in three different seed batches ($n = 150$–300): at 5 days endosperm rupture percentage is either 0 or 100% depending on the genotype and the light condition as shown in Figure 1.

was highest in *ga1* seeds and markedly lower in *ga1/rgl2* seeds, consistent with the earlier results using paclobutrazol (PAC)-treated wild type (WT) and *rgl2* seeds, and reached its lowest levels in *ga1/rgl2/gai/rga* seeds (Piskurewicz *et al*, 2008). However, *XERICO* mRNA accumulation was comparable in *ga1* and *ga1/rgl2* seeds under FR light, but not in *ga1/rgl2/gai/rga* seeds, in which it was markedly lower (Supplementary Figure 4). Taken together, these data are further consistent with the notion that *XERICO* is a target gene of the DELLA factors RGL2, GAI and RGA to promote an elevation in endogenous ABA levels during seed germination.

To discriminate between the role of DELLA factors to prevent testa rupture and their role to repress endosperm rupture by stimulating endogenous ABA levels, we treated *ga1/rgl2/gai/rga* with ABA under FR conditions. ABA-treated *ga1/rgl2/gai/rga* seeds ruptured their testa, but failed to rupture their endosperm (Figure 3A). As expected, this also correlated with higher ABI5 protein levels (Figure 3B, compare with Figure 2C). Similarly, treating *ga1* seeds with both GA and ABA led to lower DELLA factor accumulation but maintained high ABI5 protein levels (Figure 4A), consistent with the earlier results (Zentella *et al*, 2007). Under these

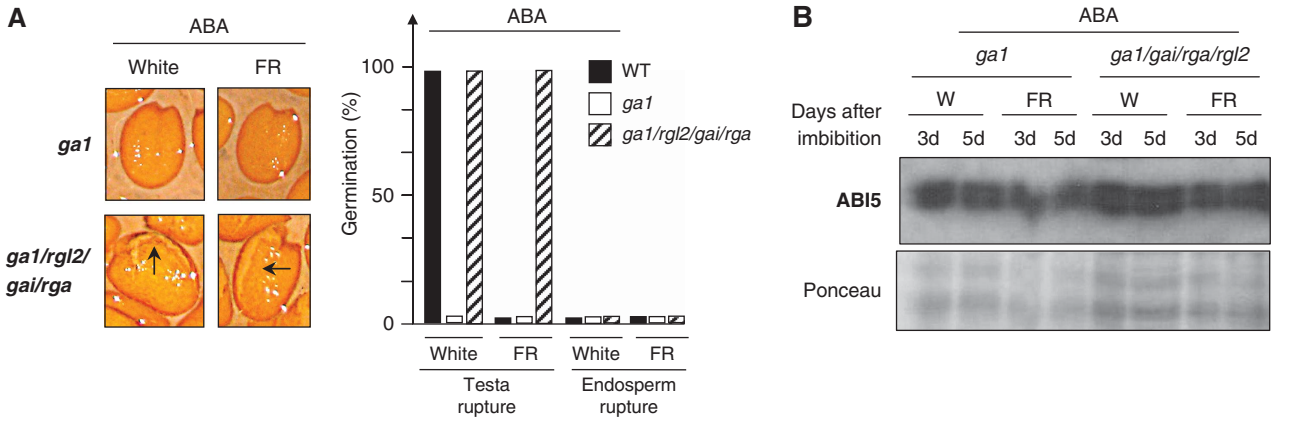

Figure 3 ABA blocks rupture of endosperm in *ga1/rga/gai/rgl2* seeds, but not that of testa. (**A**) Representative pictures show testa rupture events in *ga1-3* and *ga1-3/rga-t2/gai-t6/rgl2-1* seeds 5 days after imbibition under white (white) or far-red (FR) conditions in the presence of 3 μM ABA. Histogram shows percentage of testa and endosperm rupture events 5 days after seed imbibition. Arrows indicate testa rupture event (in three independent seed batches ($n = 150$–300) testa rupture percentage is always either 0 or 100% depending on the genotype and the light conditions as shown in the histogram). (**B**) Protein gel blot analysis of ABI5 protein levels in *ga1-3* and *ga1-3/rga-t2/gai-t6/rgl2-1* seeds at the indicated times upon imbibition under far-red (FR) or white (W) light conditions in the presence of 3 μM ABA. Protein gel blot conditions as in Figure 2C.

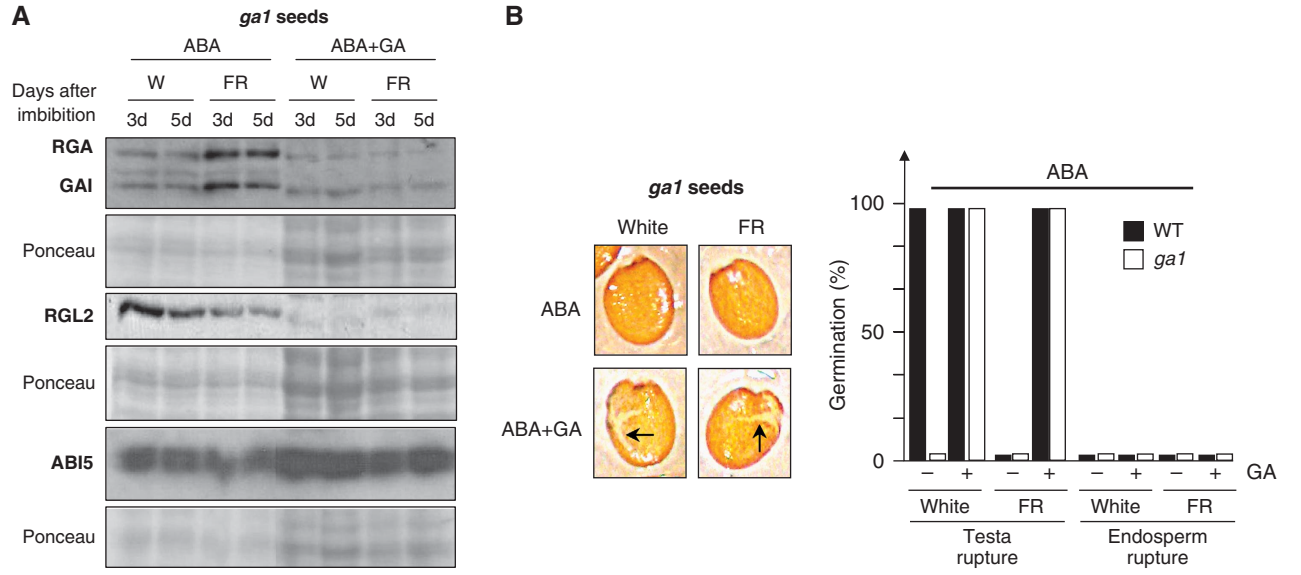

Figure 4 Exogenous GA promotes testa rupture by downregulating DELLA protein levels without overcoming ABA-dependent blockade of endosperm rupture. (**A**) Protein gel blot analysis of GAI, RGA, RGL2 and ABI5 protein levels in *ga1-3* seeds at the indicated times upon imbibition under white (W) or far-red (FR) light conditions in the presence of 3 μM ABA without (ABA) or with 50 μM GA (ABA + GA). Each lane contains proteins extracted from 5 mg of seeds. (**B**) Representative pictures showing *ga1-3* seeds after 5 days under white (white) or far-red (FR) light conditions in the presence of 3 μM ABA without (ABA) or with 50 μM GA (ABA + GA). Arrows indicate testa rupture event. Histogram shows percentage of testa and endosperm rupture events at this time point (in three independent seed batches ($n = 150$–300) testa rupture percentage is always either 0 or 100% depending on the genotype and the light conditions as shown in the histogram).

conditions, testa rupture was visible, but no endosperm rupture took place (Figure 4B).

GAI and RGA can restore normal sensitivity to low GA in rgl2 mutants

We suggested earlier that under white light conditions, RGL2 is the main factor repressing seed germination because it achieves the highest protein accumulation relative to other DELLA factors when GA levels are low. This may be due to the fact that ABA positively and strongly regulates *RGL2* mRNA levels, which is not the case for *GAI* and *RGA* (Piskurewicz *et al*, 2008). Here, we provided genetic evidence that GAI and RGA are also necessary to elevate endogenous ABA levels under FR conditions. Thus, we reasoned that both GAI and RGA should be able to substitute RGL2's function under white light conditions, provided their expression is under the control of *RGL2* promoter.

To explore this possibility, we generated *rgl2* mutant transgenic lines carrying a transgene containing either *GAI* or *RGA* coding sequences under the control of *RGL2* promoter sequences (*rgl2/pro-RGL2::GAI* and *rgl2/pro-RGL2::RGA*). We examined seed germination responses to low GA levels in independent lines using PAC, which inhibits *ent*-kaurene oxidase, a key enzyme of the GA synthesis pathway.

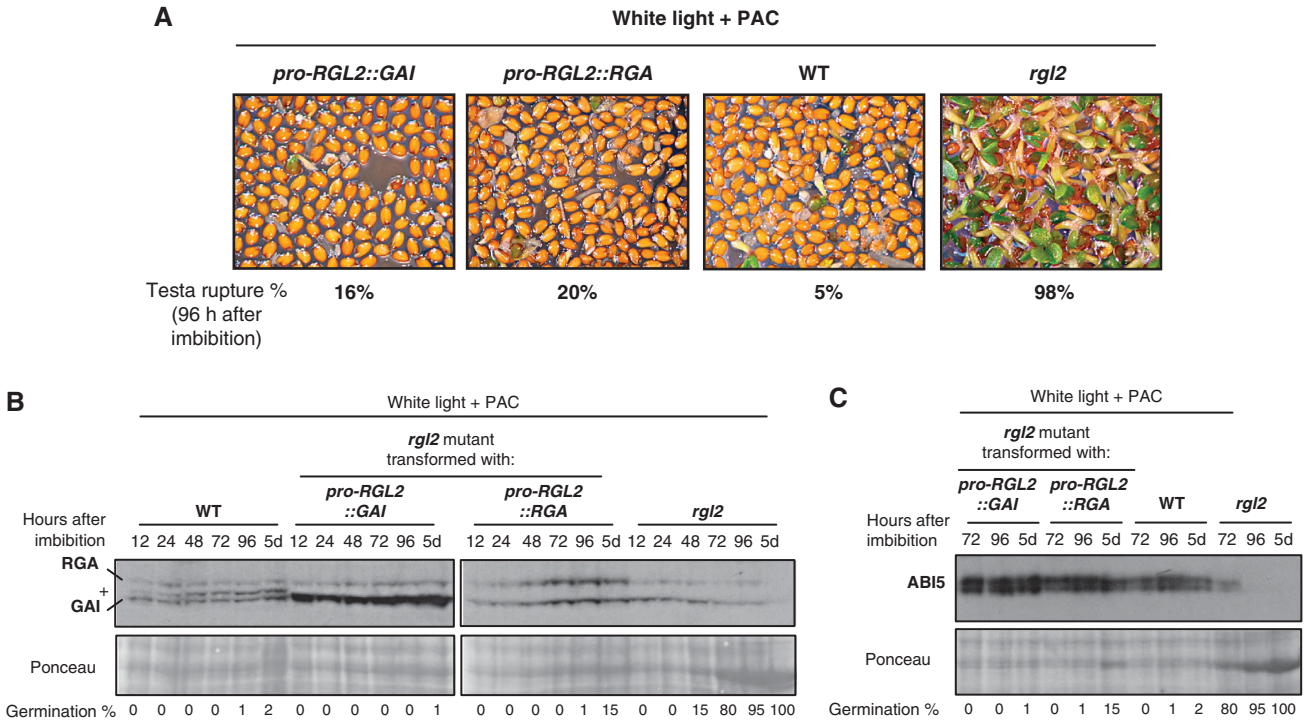

Figure 5 When expressed under *RGL2* promoter sequences, *GAI* and *RGA* can inhibit *rgl2* mutant seed germination in response to low GA levels. (**A**) Pictures show *rgl2-13* (Col) mutants transformed with *pro-RGL2*::*GAI* and *pro-RGL2*::*RGA* DNA constructs as well as *rgl2-13* and WT (Col) plants 96 h upon imbibition under white light conditions in the presence of 15 μM PAC (concerning the use of PAC, see Supplementary Figure 10). Average percentage of testa rupture events measured 96 h on imbibition is indicated (see also Supplementary Figure 5B). In the absence of PAC, all genotypes germinated similarly (not shown). (**B**) Protein gel blot analysis of GAI and RGA protein levels in WT (Col), *rgl2-13/pro-RGL2*::*GAI*, *rgl2-13/pro-RGL2*::*RGA* and *rgl2-13* seeds at the indicated time points upon seed imbibition under same conditions as in A; 10 μg of total protein is loaded per lane. The band indicated by a plus sign (+) is RGL2, which may be detected with anti-GAI antibody. At each time point, the percentage of germination (i.e. endosperm rupture events) in the seed population material used in the protein blot is indicated. (**C**) Same as in B, but ABI5 protein levels are monitored.

As expected, repression of testa and endosperm rupture could be observed in PAC-treated *rgl2/pro-RGL2*::*GAI* and *rgl2/pro-RGL2*::*RGA* transgenic lines (Figure 5A; Supplementary Figure 5B). RNA and protein blot analysis confirmed that this was associated with higher *GAI* or *RGA* mRNA and protein product levels relative to PAC-treated WT and *rgl2* seeds (Figure 5B; Supplementary Figure 5A). In addition, *rgl2/pro-RGL2*::*GAI* and *rgl2/pro-RGL2*::*RGA* arrested seeds accumulated high ABI5 protein levels, unlike *rgl2* mutant seeds (Figure 5C). This is consistent with the notion that sufficient GAI or RGA accumulation can increase endogenous ABA levels during seed germination even under white light conditions.

Taken together, these data support the hypothesis that the DELLA factors RGL2, GAI and RGA collectively repress seed germination under FR conditions by (1) repressing testa rupture and (2) stimulating endogenous ABA synthesis. In turn, higher ABA levels prevent endosperm rupture by stimulating the expression and activity of ABA-response factors, such as ABI5.

Absence of PIL5 does not prevent high GAI and RGA accumulation under low GA conditions

PIL5 is a bHLH transcription factor preferentially interacting with the active P_{fr} form of the phytochrome, which facilitates its degradation by the 26S proteasome (Oh *et al*, 2004, 2006). Under FR conditions, PIL5 is stable and stimulates the transcription of *GAI* and *RGA* (Oh *et al*, 2007). *pil5* mutants can germinate under FR conditions and this is associated with lower endogenous ABA levels. However, *pil5* mutants cannot germinate in the absence of GA synthesis (as in a *ga1* background), which is associated with increased endogenous ABA levels (Oh *et al*, 2006). This shows that PIL5 is no longer essential to repress seed germination under low GA levels. We wished to further clarify the role of PIL5 in the context of the repressive function of RGL2, GAI and RGA outlined above.

Under FR conditions, *pil5* mutants ruptured their testa before endosperm rupture (i.e. germination), unlike WT seeds (Figure 6A). Treating seeds with PAC prevented testa and endosperm rupture in both WT and *pil5* seeds (Figure 6A). These observations are consistent with the earlier results (Oh *et al*, 2006).

Direct measurement of endogenous GAI and RGA protein levels in *pil5* seeds under FR conditions was not reported earlier. Under FR conditions, we observed, as expected, that *pil5* seeds had lower endogenous RGA and GAI protein levels relative to WT, although they could still be detected (Figure 6B). In contrast, PAC treatment led to higher RGA and GAI protein levels in both WT and *pil5* seeds (Figure 6B). This may result from higher RGA and GAI protein stability as a result of lower GA levels. Critically, RGA and GAI protein levels in PAC-treated *pil5* seeds were similar to those observed in WT seeds under FR conditions in the absence of PAC treatment (Figure 6B). This observation readily provides an explanation as to why preventing GA synthesis in *pil5*

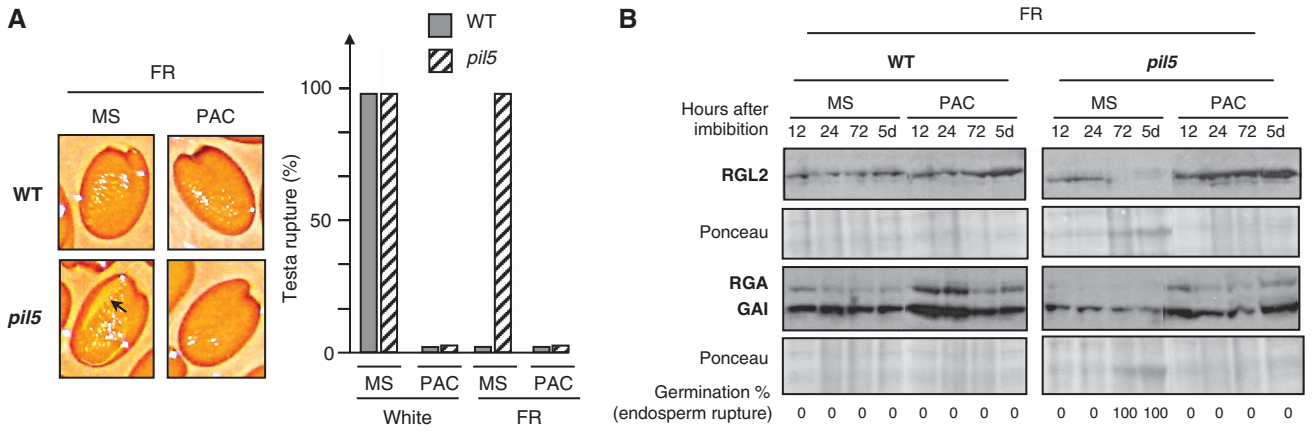

Figure 6 Absence of PIL5 does not prevent high GAI and RGA accumulation under low GA conditions. (**A**) Representative pictures show WT (Col) and *pil5* seeds 24 h upon imbibition under far-red (FR) conditions in the absence (MS) or the presence of 5 μM PAC (PAC) (concerning the use of PAC, see Supplementary Figure 10). Arrows indicate testa rupture event. Histogram shows percentage of testa rupture events 5 days upon imbibition under white (white) or far-red (FR) conditions in the absence (MS) or the presence of 5 μM PAC (PAC) (in three independent seed batches ($n = 150$–300) testa rupture percentage is always either 0 or 100% depending on the genotype and the light conditions as shown in the histogram). (**B**) Protein gel blot analysis of a time course of GAI, RGA and RGL2 protein levels in WT (Col) and *pil5* upon imbibition under far-red (FR) conditions in the absence (MS) or the presence of 5 μM PAC (PAC); 10 μg of total protein is loaded per lane. At each time point, the percentage of germination (i.e. endosperm rupture events) in the seed population material used in the protein blot is indicated.

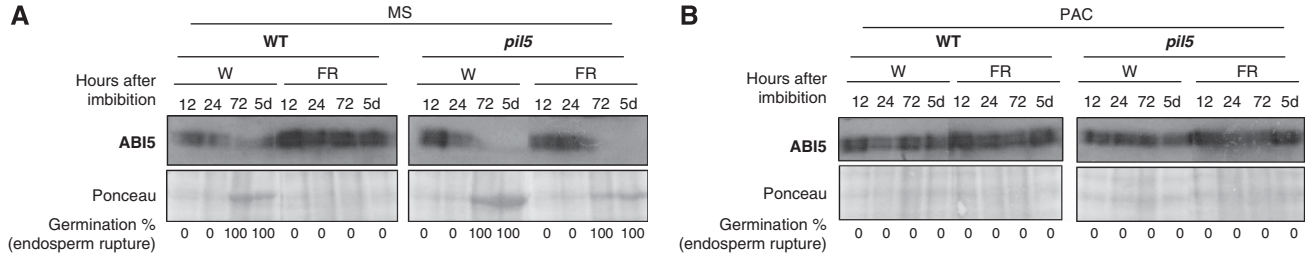

Figure 7 Absence of PIL5 does not prevent ABI5 accumulation under low GA conditions. (**A**) Protein gel blot analysis of a time course of ABI5 protein levels in WT (Col) and *pil5* seeds upon imbibition under far-red (FR) or white (W) light conditions; 10 μg of total protein is loaded per lane. At each time point, the percentage of germination (i.e. endosperm rupture events) in the seed population material used in the protein blot is indicated. (**B**) Same as in (A), but WT (Col) and *pil5* seeds were imbibed in the presence of 5 μM PAC (PAC) (concerning the use of PAC, see Supplementary Figure 10).

mutants leads to blockade of testa rupture and higher endogenous ABA levels: sufficient GAI and RGA protein levels accumulate to stimulate endogenous ABA synthesis given that they are similar to those in WT seeds under FR conditions. In turn, higher ABA levels would ensure high RGL2 protein levels by increasing *RGL2* mRNA levels (Piskurewicz *et al*, 2008). Consistent with this view, RGL2 protein levels increased in PAC-treated *pil5* seeds (Figure 6B). In the absence of PAC treatment, RGL2 protein levels were roughly similar between WT and *pil5* seeds at early time points after imbibition (12, 24 h), but rapidly decayed thereafter in *pil5* (Figure 6B). Thus, failure to maintain RGL2 protein levels is consistent with the fact that *pil5* have low endogenous ABA levels rather than the result of the absence of PIL5. Indeed, PIL5 does not seem to directly regulate *RGL2* transcription (Oh *et al*, 2007), whereas ABA strongly upregulates RGL2 mRNA levels (Piskurewicz *et al*, 2008).

Furthermore, WT seeds under FR conditions accumulated high ABI5 protein levels relative to white light conditions and could not germinate (Figure 7A). In contrast, *pil5* mutants, which accumulate low GAI, RGA and RGL2 levels (Figure 6B), failed to maintain high ABI5 protein levels and germinated (Figure 7A). As expected, PAC-treated *pil5* seeds, which accumulate high levels of GAI, RGA and RGL2 (Figure 6B), maintained high ABI5 protein levels and did not germinate (Figure 7B). These results are consistent with the notion that GAI and RGA factors stimulate endogenous ABA levels under FR conditions.

ABI3 is the main factor repressing germination under FR conditions

We further evaluated the role of ABA to repress seed germination under FR conditions when GA synthesis is prevented. If RGL2, GAI and RGA prevent endosperm rupture by promoting an increase in endogenous ABA levels, then preventing ABA synthesis should lead to seed germination, as reported earlier under white light (Leon-Kloosterziel *et al*, 1996; Piskurewicz *et al*, 2008).

Unexpectedly, PAC-treated *aba1* mutant seeds, unable to synthesize ABA, germinated poorly (20% after 5 days) under FR conditions (Figure 8; Supplementary Figure 7) even though they accumulated lower RGL2 and ABI5 protein levels relative to WT seeds, consistent with earlier results (Piskurewicz *et al*, 2008) (Supplementary Figure 8). However, we found that *aba1* seeds did not rupture their testa, consistent with their high RGA and GAI protein

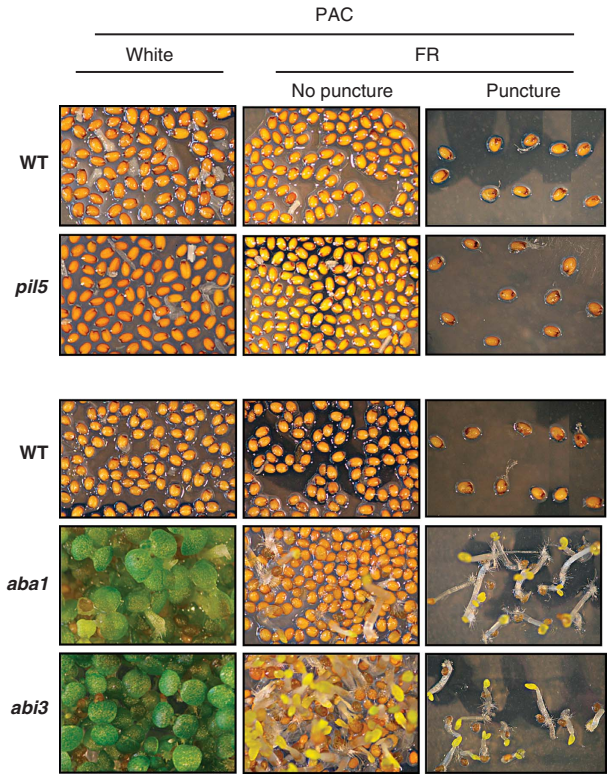

Figure 8 ABA acts through ABI3 to inhibit endosperm rupture in FR conditions. Pictures show WT (Col), *pil5*, WT (Ler), *aba1-1* and *abi3-1* seeds 7 days upon imbibition under white (white) or far-red (FR) conditions in the presence of 5 μM PAC (PAC) (concerning the use of PAC, see Supplementary Figure 10). Seeds were used for puncture experiments (Materials and methods). Punctured seeds were transferred to normal medium (MS) under white light conditions to assess seed viability after surgical procedure (Supplementary Figure 6). The procedure did not prevent seed germination (Supplementary Figure 6 shows WT (Col and Ler) and *pil5* seeds after puncture).

accumulation, which was similar to that of WT seeds (Figure 9A). Thus, we reasoned that failure of *aba1* seeds to rupture their testa might mechanically block radicle elongation, therefore, preventing the visualization of endosperm rupture (i.e. defined here as visible radicle protusion out of the seed coat). To address this possibility, we punctured *aba1* seeds with a needle before FR irradiation (Material and methods). This surgical procedure triggered germination in each PAC-treated *aba1* mutant seed (Figure 8; Supplementary Figures 6 and 7). Seed germination could not be observed in punctured PAC-treated WT or *pil5* mutant seeds (Figure 8; Supplementary Figures 6 and 7). Control experiments performed in parallel showed that the puncture assay does not kill embryos (Supplementary Figures 6 and 7).

Finally, we wished to identify key ABA-response factors repressing endosperm rupture under FR conditions. An obvious candidate is ABI5, which indeed is necessary to repress endosperm rupture under white light conditions (Piskurewicz *et al*, 2008). However, PAC-treated *abi5* mutants germinated poorly under FR conditions, even after mechanical puncture (Supplementary Figures 6 and 7). This could be due to higher endogenous ABA levels under FR conditions, resulting from the concerted activity of GAI, RGA and RGL2. In turn, higher ABA levels may increase the activity of additional ABA-response factors. Given that ABI3 is known to act upstream of ABI5 during seed germination in response to ABA (Lopez-Molina *et al*, 2002), we reasoned that it may also control the expression of additional ABA-response repressors of seed germination. Indeed, we observed 20–80% seed germination in PAC-treated *abi3-1* (Ler ecotype) seeds depending on the seed batch (Figure 8). We also examined a weak *abi3* mutant allele (*abi3-9*, Col ecotype) seed population, in which 0% of seed germination took place under FR conditions (Supplementary Figure 7). We noticed that *abi3-1* or *abi3-9* seed germination took place without prior visible testa rupture, similar to what is observed in PAC-treated *aba1* mutant seeds under FR conditions (Figure 8). Consistently, *abi3-1* accumulated high GAI and RGA protein levels under FR conditions (Figure 9A). Thus, we reasoned that failure to rupture testa may also mask endosperm rupture events in *abi3-1* and *abi3-9* seeds. Consistent with this hypothesis, puncturing *abi3-1* and *abi3-9* seeds strongly promoted germination (i.e. radicle elongation) in each case (Figure 8; Supplementary Figure 7).

We also monitored *ABI3* expression in *ga1*, *ga1/rgl2* and *ga1/rgl2/gai/rga* seeds under white and FR light conditions. Figure 9B shows that under white light conditions *ABI3* mRNA expression was maintained over time in *ga1* seeds, but not in *ga1/rgl2* and *ga1/rgl2/gai/rga* seeds. Under FR light conditions, *ABI3* mRNA expression was maintained in *ga1* and *ga1/rgl2* seeds, but not in *ga1/rgl2/gai/rga* seeds (Figure 9B). Similarly, high *ABI3* mRNA expression was maintained in PAC-treated WT, *rgl2/pro-RGL2::GAI* and *rgl2/pro-RGL2::RGA* transgenic lines, but not in PAC-treated *rgl2* mutants under white light conditions (Figure 9C). Collectively, these data are consistent with the notion that DELLA-dependent accumulation of endogenous ABA levels stimulates *ABI3* expression.

In conclusion, our observations strongly support the notion that under FR conditions, only ABA levels determine the potential of a seed to rupture its endosperm (i.e. defined here as visible radicle protusion out of the seed coat), irrespective of GA levels. In contrast, DELLA factors have an important function to repress testa rupture and to promote higher endogenous ABA levels. This conclusion strengthens and extends our earlier studies of seed germination under white light conditions.

Discussion

In the present work, we assessed the role of the GA- and ABA-response factors under FR light conditions ensuring phyB inactivity. The salient findings of this study are (1) in the absence of GA synthesis, RGA, GAI and RGL2 act to (a) repress testa rupture and (b) repress endosperm rupture by increasing endogenous ABA levels, which in turn inhibit endosperm rupture through ABI3 and (2) to put the role of PIL5 in a new perspective: PIL5 ensures sufficient GAI and RGA protein accumulation under FR conditions to block testa rupture and promote high endogenous ABA levels. However, the role of PIL5 is restricted to FR conditions and can be overshadowed under conditions in which GA synthesis is severely prevented (as in PAC-treated *pil5* seeds or in *pil5/ga1* seeds). In this case, sufficient GAI and RGA accumulation occurs, so that testa and endosperm rupture is prevented despite the absence of PIL5. Thus, this work extends and

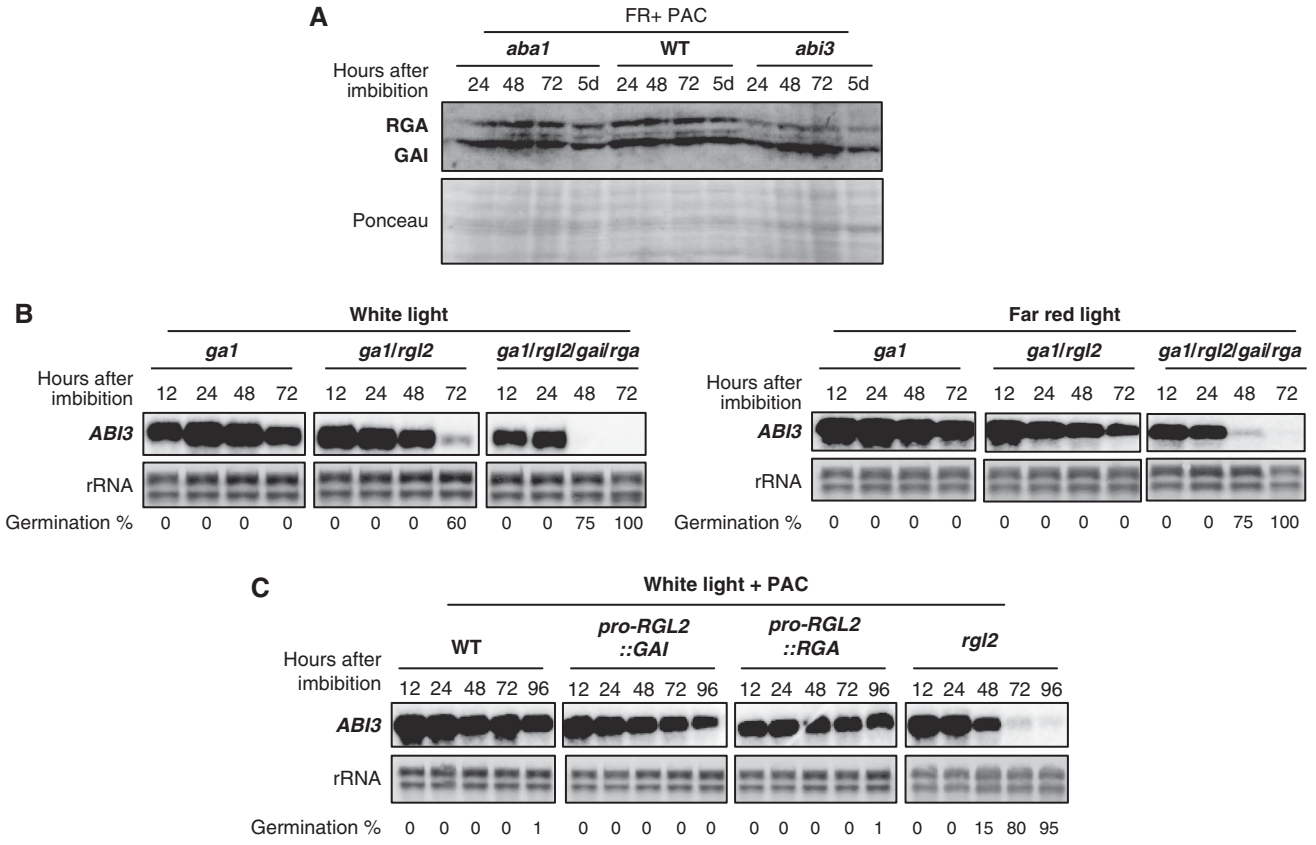

Figure 9 *aba1* and *abi3* seeds accumulate WT-like levels of GAI and RGA consistent with their inability to rupture the testa. DELLA-dependent increase in endogenous ABA levels correlates with high *ABI3* mRNA expression. (**A**) Protein gel blot analysis of RGA and GAI protein levels in WT (Ler) and *aba1-1* and *abi3-1* seeds at the indicated times upon seed imbibition under far-red conditions in the presence of 5 μM PAC (FR + PAC) (concerning the use of PAC, see Supplementary Figure 10); 10 μg of total protein is loaded per lane. (**B**) RNA gel blot analysis of the time course of *ABI3* mRNA accumulation in *ga1-3*, *ga1-3/rgl2-13* and *ga1-3/rga-t2/gai-t6/rgl2-1* seeds under white or far-red light conditions (normal MS medium). Two micrograms of total RNA was used per lane. rRNA is shown as a loading control. At each time point, the percentage of germination (i.e. endosperm rupture events) in the seed population material used in the RNA blot is indicated. (**C**) RNA gel blot analysis of the time course of *ABI3* mRNA accumulation in PAC-treated (15 μM) WT (Col), *pro-RGL2*::*GAI*, *pro-RGL2*::*RGA* transgenic lines and *rgl2-13* seeds under white light conditions. Two micrograms of total RNA was used per lane. rRNA is shown as a loading control. At each time point, the percentage of germination (i.e. endosperm rupture events) in the seed population material used in the RNA blot is indicated.

confirms our recent studies on the control of Arabidopsis seed germination under white light conditions. Below, we put these findings in the perspective of the earlier studies.

The antagonistic effects that ABA and GA have on Arabidopsis seed germination have long been established. After seed imbibition, ABA levels, high in the mature seed, drop rapidly, a process most likely involving CYP707 hydroxylases (Okamoto *et al*, 2006). In parallel, GA levels start to rise as soon as 12–16 h on imbibition (Ogawa *et al*, 2003). Preventing GA synthesis in seeds or exposing them to ABA will block their germination. Similarly, environmental conditions that stimulate seed germination will tend to be associated with high GA levels and low ABA levels, whereas conditions unfavourable with seed germination will be associated with low GA levels and high ABA levels (Olszewski *et al*, 2002; Nambara and Marion-Poll, 2005).

Taken together, these observations have led to the view that it is the balance of ABA and GA levels that determines the germination potential of the seed (Seo *et al*, 2009). In this view, GA and ABA control the expression and activity of their respective response factors that then independently act to control germination. However, closer inspection indicates that this representation can be misleading. Indeed, this is because it may suggest that (1) GA and ABA levels exert their influence on the same developmental steps during germination and (2) it is only the ratio of GA to ABA that determines the germination potential of a seed. Moreover, another consideration is often overlooked: conclusions about the role of GA and ABA in controlling seed germination may be flawed depending on the developmental state of the seed, that is commitment to germinate or not. We further discuss these issues below.

GA and ABA exert striking different influences on Arabidopsis early germination processes (Muller *et al*, 2006; Piskurewicz *et al*, 2008). Seed germination involves a succession of developmental processes, notably, at an early stage, testa rupture, which is followed after a few hours by the rupture of the endosperm (defined here as the earliest visible protrusion of the radicle out of the testa) (Muller *et al*, 2006; Piskurewicz *et al*, 2008). The latter step is usually chosen to assess whether seed germination has taken place, but this completely overlooks testa rupture. Indeed, depending on the germination conditions and the genetic background, visible testa rupture may not take place before endosperm rupture

(e.g. as in PAC-treated *abi5* mutants) (Piskurewicz *et al*, 2008). A seed arrested because of low GA levels does not have the same appearance as a seed arrested because of high ABA levels. Indeed, in *ga1-3* seeds, both testa and endosperm rupture is completely blocked. In contrast, in ABA-treated WT seeds, testa rupture is only delayed, which contrasts with the robust blockade of endosperm rupture (Muller *et al*, 2006; Piskurewicz *et al*, 2008). As a result, all ABA-arrested seeds eventually have a ruptured testa, although the embryo remains surrounded by the endosperm. This indicates that ABA blocks endosperm weakening and radicle elongation. Moreover, even high GA concentrations (e.g. 50 μM) cannot overcome relative low ABA concentrations that are sufficient to prevent endosperm rupture (e.g. 3 μM) (Supplementary Figure 9).

These observations strongly suggest that GA synthesis favours germination mainly because it promotes testa rupture. In contrast, ABA mainly represses endosperm rupture irrespective of GA levels. These considerations are not easy to reconcile with the view that the ratio of GA to ABA levels defines the germination capacity. The observation that low GA levels in seeds can lead to germination when ABA levels are low (as in an PAC-treated *aba1* seeds) (Leon-Kloosterziel *et al*, 1996; Piskurewicz *et al*, 2008) can be interpreted as follows: lack of testa rupture when GA levels are low is associated with high endogenous ABA, which prevents endosperm rupture (Piskurewicz *et al*, 2008). When ABA synthesis is prevented (as in *aba1*), endosperm rupture may take place, provided the mechanical resistance of the unruptured testa is overcome by the embryo (Piskurewicz *et al*, 2008).

The evidence that PIL5 and SOMNUS repress germination by altering, in an unknown manner, the expression of GA and ABA metabolic genes is rather circumstantial. Indeed, it is based on the observation that *pil5* and *som* seed germination under FR conditions is associated with high GA and low ABA levels and concomitant changes in GA and ABA metabolic gene expression. In this context, it is significant to note that no direct regulation of metabolic gene expression by PIL5 or SOM has been evidenced so far (such as PIL5 or SOM binding to promoter elements of metabolic genes) (Oh *et al*, 2007; Kim *et al*, 2008). Without further investigation, a given state of metabolic gene expression or hormone levels may be interpreted as the consequence rather than the cause of seed germination. This underlies the difficulty to assess the relative role of GA and ABA in controlling seed germination. In particular, there is also little direct evidence available to support the proposed function of ABA and ABA-responses factors to repress seed germination under FR conditions (Seo *et al*, 2009). To better understand the role of ABA, we chose to keep GA levels always low, such as in a *ga1* seeds or PAC-treated seeds. In this case, testa and endosperm rupture is prevented. We could then examine the role of DELLA factors, ABA and ABA-response factors in controlling testa and endosperm rupture, thus allowing to establish causal effects.

In Piskurewicz *et al*, we argued that under white light conditions GA controls the levels of ABA through RGL2 (Piskurewicz *et al*, 2008). Indeed, when GA levels are low, a stabilized RGL2 stimulates endogenous ABA levels, a process that may involve direct stimulation of *XERICO* transcription by RGL2 (see below) (Supplementary Figure 4) (Zentella *et al*, 2007; Piskurewicz *et al*, 2008). *XERICO* encodes a putative RING-H2 zinc-finger factor increasing endogenous ABA levels in an unknown manner (Ko *et al*, 2006; Zentella *et al*, 2007). It is highly relevant to mention here that *XERICO* was first proposed to be a target gene of GAI and RGA in seedlings, in which both were shown to bind *XERICO* promoter sequences (Zentella *et al*, 2007). In turn, in seeds, higher endogenous ABA, through its positive control of ABI5 protein levels and activity, is the main, if not only variable, determining the potential of the seed to rupture endosperm. Equally important is the fact that endogenous ABA is also essential to stimulate high *RGL2* mRNA levels, thus ensuring a positive feedback loop to maintain high RGL2 and, therefore, high ABI5 protein levels to ensure repression of endosperm rupture (Piskurewicz *et al*, 2008). On the other hand, GA levels are essential, through RGL2, to determine the potential for testa rupture. Indeed, ABA has a moderate influence on testa rupture. These conclusions are mainly based on the observation that *rgl2* mutants have low ABA and low ABI5 protein levels when GA levels are low. Moreover, the converse is not true: when GA levels are low, *abi5* mutants have high RGL2 protein levels and cannot break the testa. This suggests that ABI5 acts downstream of RGL2 and that both factors do not repress germination independently and in parallel as proposed earlier (Oh *et al*, 2007; Kim *et al*, 2008; Seo *et al*, 2009).

Here, we show that this view can be generalized under FR conditions, the main difference being that RGA and GAI participate together with RGL2 to repress testa rupture and to elevate endogenous ABA levels. Thus, only *ga1/rgl2/gai/rga* can rupture testa and accumulate low levels of ABA and ABA-response factors under FR conditions. In contrast, *aba1* and *abi3* mutants maintain high DELLA factors and do not rupture testa (Figure 9A), consistent with the notion that they act downstream of DELLA factors. We showed that in this case, it is necessary to surgically puncture seeds to unveil the capacity of PAC-treated *aba1* and *abi3* seeds to germinate (i.e. in this case, to elongate their radicle as puncture experiments rupture both testa and endosperm).

Our work provides a new perspective for PIL5 and its role to regulate *GAI* and *RGA* expression. Under white light or red light, the role of PIL5 is irrelevant, as the P_{fr} form of phyB triggers its degradation. In contrast, PIL5 accumulates under FR conditions and was proposed to positively regulate *GAI* and *RGA* transcription (Oh *et al*, 2007). Consistently, we confirm that endogenous GAI and RGA protein levels are lower in *pil5* under FR conditions. However, we show that in the absence of GA synthesis (as in PAC-treated *pil5* seeds), the role of PIL5 becomes marginal as GAI and RGA accumulate at levels similar to that of WT seeds under FR conditions. This offers a simple explanation as to why *ga1/pil5* seeds do not rupture testa and endosperm: they produce sufficient GAI, RGA and RGL2 protein accumulation to block testa rupture and to produce sufficient ABA accumulation. Consistent with this view, surgical rupture assays in PAC-treated *pil5* seeds do not lead to germination (i.e. radical elongation).

This raises the possibility that *pil5* seed germination under FR conditions is only because of low GAI and RGA levels. This is unlikely, as PIL5 also positively regulates the transcription of *SOM*, which is indirectly involved in promoting ABA synthesis (Kim *et al*, 2008). SOM regulates GA and ABA

metabolic genes indirectly, but it is not expected to do so through GAI and RGA, as it does not regulate *DELLA* transcript accumulation (Kim *et al*, 2008). Thus, it is plausible that GAI, RGA, RGL2 and SOM collectively contribute to raise endogenous ABA levels under FR conditions. Moreover, GAI and RGA promotion of higher endogenous ABA levels does not seem to be restricted to seed germination under FR conditions. This is substantiated by the observation that *GAI* and *RGA* can substitute for *RGL2's* function under white light conditions by restoring high ABI5 protein levels and hence blocking germination (Figure 5). Thus, the positive regulation of *GAI* and *RGA* mRNA levels by FR light through PIL5 can be viewed similarly to ABA's positive regulation of *RGL2* mRNA levels: it is essential to ensure sufficient GAI and RGA protein levels, so that they promote high endogenous ABA levels under FR conditions (see model Figure 10). This underlines the fact that the role of DELLA genes in plant growth and development cannot be solely described or understood from the perspective of DELLA protein stability, but necessitates an understanding of how mRNA levels are regulated.

Finally, it remains to be understood which are the DELLA-dependent mechanisms that lead to higher endogenous ABA levels when GA levels are low. This necessitates the understanding of the molecular function of DELLA factors, which is still uncertain. Recent reports have suggested that DELLA factors inhibit hypocotyl elongation by preventing PIL/PIF factors to bind target genes involved in cellular elongation, such as *LTP3* and *Expansin* (Feng *et al*, 2008; de Lucas *et al*, 2008). In the case of *XERICO*, Zentella *et al* showed that *XERICO* promoter DNA sequences were enriched in ChIP experiments looking for RGA target genes in seedlings (Zentella *et al*, 2007). Our data are consistent with the proposition that DELLA factors may stimulate the transcription of *XERICO* to promote ABA synthesis to block germination (Zentella *et al*, 2007). Whether this involves the interaction with a bHLH transcription factor is speculative at the present time. Here, we have considered the case of the bHLH transcription factor PIF1/PIL5, whose proposed function is to activate the transcription of *RGA* and *GAI*. This is supported by genetic evidence and ChIP data (Oh *et al*, 2007). However, in the case of PIL5, there is no biochemical or genetic evidence that PIL5 activity is dependent on one or several DELLA factors (e.g. no interaction between PIL5 and a DELLA factor has been evidenced so far). Thus, although no direct DNA-binding activity of a DELLA factor has been shown, the evidence so far suggests that DELLA factors stimulate or repress gene transcription to exert their developmental role. However, it should be noted that seed germination is a rather peculiar process during which ABA has a central and temporally restricted regulatory function (Lopez-Molina *et al*, 2001; Piskurewicz *et al*, 2008). The regulation of hypocotyl elongation occurs well after the developmental window, in which the plant growth is highly sensitive to ABA. We have performed medium shift experiments in darkness that indicate that ABA poorly inhibits hypocotyl elongation in the dark (UP and LLM, unpublished observations). This could suggest that DELLA-dependent inhibition of hypocotyl elongation does not involve an increase in endogenous ABA levels. Although it seems highly likely that DELLA-dependent stimulation of *XERICO* expression is involved in promoting endogenous ABA synthesis, this does not exclude additional DELLA-dependent processes that do not involve changes in gene transcription. Thus, DELLA factors may regulate ABA metabolism by interacting with proteins other than transcription factors, by perhaps interfering with the activity of proteins regulating ABA metabolism.

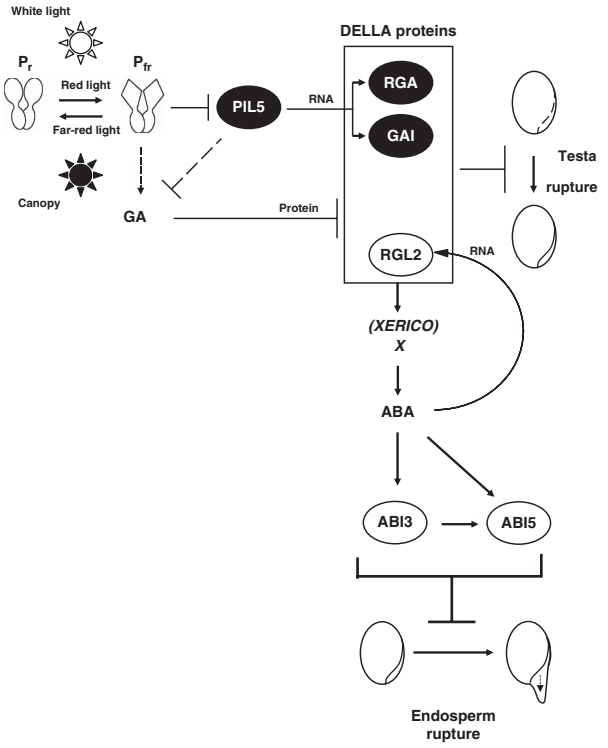

Figure 10 A model for the control of seed germination under canopy conditions. Upon seed imbibition, canopy conditions (FR conditions) inactivate phytochromes, thus repressing GA synthesis in an unknown manner while stabilizing PIL5, which also represses GA synthesis indirectly. In turn, PIL5 stimulates *GAI* and *RGA* transcription, which ensures that sufficient GAI and RGA protein will accumulate to elevate, together with RGL2, endogenous ABA levels. PIL5, GAI and RGA are represented by black ovals to emphasize that they are relevant only under FR conditions. DELLA proteins repress seed germination by (1) blocking testa rupture and (2) stimulating ABA levels, which may involve stimulation of *XERICO* expression as well as other unidentified factors (X). In turn, ABA blocks endosperm rupture by stimulating ABI3 and ABI5 expression and product activity. RGL2 (white oval) has a central role to repress germination because *RGL2* mRNA levels are positively regulated by ABA. This ensures high RGL2 protein levels irrespective of light conditions (i.e. FR or white light conditions).

Materials and methods

Plant material

Throughout this study, we use non-dormant seeds in the absence of seed stratification procedure. We refer to seeds sown under 'normal conditions' when seeds are imbibed in a standard germination medium (MS).

The *Arabidopsis* mutants *aba1-1* ('*aba1*' in the text) and *ga1-3* (Ler, *ga1*) were obtained by Marteen Koornneef. *rgl2-13* is in the Columbia (Col-0) background (Tyler *et al*, 2004) and *ga1-3/rgl2-13* (*ga1/rgl2*) seeds were obtained from TP Sun. *ga1-3/rgl2-1/gai-t6/rga-t2* (Ler, *ga1/rgl2/gai/rga*) were obtained by NP Harberd. The *abi5-3* (Col-0 ecotype) mutant was obtained from RR Finkelstein (Finkelstein and Lynch, 2000). *abi3-1* (Ler) was obtained by F Parcy and *abi3-9* (Col) by E Nambara (Nambara *et al*, 2002). *pil5* (*pif1-2*) was obtained from C Fankhauser and first described in Huq *et al* (2004). Supplementary Figure 11 lists seed ecotypes and donors.

Germination assays

All seed batches compared in this study were harvested on the same day from plants grown side by side (i.e. identical environmental conditions). Dry siliques were obtained about 8 weeks after planting and left for a further 4 weeks at room temperature before seed harvesting. Seeds were then permanently stored at 4°C. Seeds obtained in this manner lacked dormancy. A minimum of three independently grown seed batches were used for measuring the per cent of testa and endosperm rupture events. For each seed batch, a minimum of two replication experiments was performed.

For germination assays, seeds were surface sterilized as described (Lopez-Molina and Chua, 2000) and sown in plates with MS medium containing 0.8% (wt/vol) Agar (Applichem). Medium was supplemented with GA_3 (G7645, Sigma), ABA (A1049, Sigma) or PAC (46046, Riedel-de Haen) according to the germination condition examined. Plates were incubated in a climate-controlled room (20–25°C, 16 h light/day, light intensity 80 $\mu E/m^{-2}s^{-1}$, humidity 70%). Between 150 and 300 seeds were examined with a Stemi 2000 (Zeiss) stereomicroscope and photographed with a high-resolution digital camera (Canon Power G6, 7.1 Megapixels) at different times of seed imbibition. Photographs were enlarged electronically for measurement of testa and endosperm rupture events.

Light treatment and surgical procedure

After sterilization, seeds were incubated in the dark for 1 h before irradiation for 5 min with FR light (2 $\mu mol\,m^{-2}\,s^{-1}$). Irradiation was performed in a growth chamber (CLF Plant Climatics, Percival CU-36L5) with 740 nm LEDs (L735-03AU, Epitex Inc, Japan). Seeds were kept in darkness by wrapping plates in several layers of aluminium foil.

The puncture procedure (using a needle) was performed 20 min after sterilization before 1 h incubation in the dark followed by FR light treatment. In a typical experiment, 15–19 seeds are punctured under white light illumination under the stereomicroscope. The procedure takes between 5 and 10 min. Thereafter, 10–14 seeds are subject to FR light irradiation, whereas five seeds are grown under normal white light illumination (positive control for seed viability). Figure 8 shows a typical example of 10 punctured seeds. These experiments were performed twice for each genotype. The seed viability test is always positive (5/5) (Supplementary Figures 6 and 7). PAC-treated WT and *pil5* seeds never elongate the radicle after the puncture procedure, whereas an average of more than 90% of *aba1* and *abi3* seeds do elongate (>90%). The results are compiled in a table in Supplementary Figure 7.

Plasmid constructs and plant transformation

DNA manipulations were performed according to standard methods (Sambrook *et al*, 1989). *prom-RGL2*::*GAI* and *prom-RGL2*::*RGA* transgene construction: *GAI* coding sequences were amplified with 5′-ATAGGCGCGCCATGTATCCATATGACGTGCCGGACTACGCCTCCC TCATGAAGAGAGATCATCATCATCATC and 5′-GCTTAATTAACTAA TTGGTGGAGAGTTTCCAAGCCGAGG and *RGA* coding sequences with 5′-ATAGGCGCGCCATGTATCCATATGACGTGCCGGACTACGCCT CCCTCATGAAGAGAGATCATCACCAATTCC and 5′-GCTTAATTAAT CAGTACGCCGCCGTCGAGAGTTTCC. In each case, the first primer contains an Asc I site and the HA sequence (MYPYDVPDYASL), whereas the second primer contains a Pac site. Both restriction sites were used for cloning into pBA002a, a promoterless version of pBA002 (Kost *et al*, 1998) lacking the 35S promoter of *Cauliflower mosaic virus*, containing approximately 2 kbp of upstream genomic promoter sequences of *RGL2* amplified with 5′-ATAGGCG CGCCCTTTCTTGTTCTTGTGATGGTGAGTTAAG and 5′-ATAGGCGC GCCGTCTCAACAGTCTCATGCCGAGATGATGG. Transgenic *Arabidopsis* lines were generated using the *Agrobacterium tumefaciens* vacuum-infiltration method (Bechtold and Pelletier, 1998). Seeds (T1) from infiltrated plants were plated in selection medium as described (Lopez-Molina *et al*, 2001).

Antibody production, protein and RNA blot analysis

The antibodies and their use for protein gel blot analysis were as described in Piskurewicz *et al* (2008). RNA blot analysis and probes are as described earlier (Piskurewicz *et al*, 2008). A *NCED6* DNA probe was generated using the following primers: 5′-CTTCTT CCGACGAAGACTTCTCC and 5′-CCGTCCGATCGTCTCAAGATCTCC.

ABA analysis

The seeds (10–100 mg of each sample f.w.) were ground using 3-mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany) with an MM 301 vibration mill at a frequency of 27.5 Hz for 3 min (Retsch GmbH & Co.). Internal standard (50 pmol of (+)-3′,5′,5′,7′,7′,7′-$^2H_6$-ABA) and 1 ml cold methanol/water/acetic acid (80/19/1, v/v) were added to the samples. After 24 h of shaking in the dark at 4°C, the homogenates were centrifuged (20 000 r.p.m., 5 min, 4°C) and the pellets were then re-extracted in 0.5 ml extraction solvent for 60 min. The supernatants were transferred to a fresh glass tube and dried under vacuum. Extracts were dissolved in 100 µl 99% methanol:1% acetic acid (v/v) topped up to 1 ml with 99% water:1% acetic acid (v/v) and purified by solid-phase extraction on an Oasis HLB cartridges (60 mg, 3 ml, Waters, Milford, MA, USA). The fraction containing ABA was eluted with 3 ml methanol/water/acetic acid (80/19/1, v/v) and evaporated to dryness in a Speed-Vac (UniEquip). Subsequently, the evaporated samples were methylated, purified by ABA-specific immunoaffinity extraction (Hradecka *et al*, 2007) and analysed by UPLC-ESI(+)-MS/MS (Turečková *et al*, unpublished).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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