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

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 April 2009

Thank you for submitting your manuscript for consideration to The EMBO Journal. I do apologize for the slight delay in getting back to you with a decision that was caused by initial difficulties in assigning suitable referees and some delay in receiving their comments. As you will see from the enclosed assessments, the referees appreciate the mostly genetic delineation of molecular pathways controlling seed germination under far-red light conditions. It also becomes obvious from the explicit reports, that certain improvements/clarifications are needed that will have to be incorporated during a single round of major amendments. This will have to include significant additional experimental work (as mostly expressed by the comments of ref#3) and more precise introduction/discussion of the available literature. If possible and along the line of ref #2' remark known direct molecular links between the players involved should at least be emphasized if not further explored. Given the overall rather supportive assessments but also aware of considerable work that will have to be done to eventually reach the rather high standards of our journal we would like to offer you the changes to improve and modify the current dataset during major revisions.
I also have to remind you that it is usually EMBO_J policy to allow a single round of revisions only, which means that the decision on acceptance or rejection entirely depends on the content within the final version of your manuscript.

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

This manuscript is a follow-up work from the previous report by the same group, "The Gibberellic Acid Signaling Repressor RGL2 Inhibits Arabidopsis Seed Germination by Stimulating Abscisic Acid Synthesis and ABI5 Activity; Plant Cell, Piskurewicz et al., 2008". In this article, authors showed that two additional DELLA proteins, RGA and GAI, also play a role in the regulation of ABA synthesis, linking between GA and ABA in the regulation of seed germination. Furthermore, authors also proposed that at least a part of effects by far-red lights in the seed germination is GA dependent, which is somewhat well-known. Authors utilized existing mutants to address the function of GA and far-red light effects (No or low GA and No active PHYB photoreceptor) to clearly see the functional relationship between DELLA proteins and ABA. Although this manuscript reported an interesting insight on the regulatory network among photoreceptors, GA, PIL5, DELLA proteins and ABA, there are several questions need to be addressed.

Major points.

1) Although title of this manuscript include "DELLA-dependent enhancement of ABA synthesis", authors did not measure the level of ABA. Authors rely on ABI5 activity for endogeneous levels of ABA. Authors should measure endogenous ABA contents in WT, ga1, and ga1/rga/gai/rgl2 mutant lines as they did so for the previous report, especially authors are reporting different sets of mutants here. Also, it will be interesting to see if any of ABA biosynthetic genes are affected in the mutants tested.

2) Also in the title, authors argue that "DELLA proteins enhance ABI3 activity". But, no direct data to show the case a) Is ABI3 expression could be affected in della mutants? b) Can DELLA overexpression line enhance the expression of ABI3 gene and ABI3-mediated downstream target genes?

3) Authors mentioned that DELLAs act upstream of ABIs (ABI3 and ABI5) through enhancement of ABA synthesis, to prove this hypothesis, Authors should measure the expression level of DELLA genes, ABI3 and ABI5 genes under -ABA and +ABA condition.

4) Authors only focused on inactive PHYB condition irradiated with far-red light for the seed germination assay. Even PHYB is the main photoreceptor regulating seed germination, PHYA photoreceptor is involved in seed germination as well (Kim et al., 2008; Plant Cell and etc.). Prolonged far-red light irradiation could stabilize PHYA photoreceptor and activated PHYA can also induce the seed germination. Thus authors should do the PHYA-mediated seed germination test. It will be useful to gain the insight on overall relationship between DELLA proteins and Phytochromes in the seed germination.

5) Although XERICO is previously reported as a target of GAI and RGA, there is no experiment on XERICO in the seed germination condition in this manuscript.

6) A model proposed in Fig.9 shows that PHYs can regulate GA directly and that PIL5 only regulates RGA and GAI. It is not consistent with other reports. There is no evidence on PHYs can directly regulate GA synthesis (even in canopy condition). It has been reported that PIL5 can regulate GA biosynthetic genes, as authors indicated in the text.

Minors
- All germination assay should include the WT control.
- In INTRODUCTION part, page 5. SOM is not bHLH protein, but a C3H zinc finger protein

Referee #2 (Remarks to the Author):

This manuscript is concerned with the role of three functionally redundant DELLA repressors (RGL2, GAI, RGA) in the control of seed germination in Arabidopsis. The authors address the role of these DELLA repressors in a far-red light enriched condition as it is present under a leaf canopy, a condition which is unfavorable for germination and plant growth for most plants. The authors show that unlike in wild type conditions where RGL2 is the major DELLA repressor of seed germination, the three above-mentioned DELLA repressors act in concert to repress germination in

far-red light enriched environment where the phyB photoreceptor is activated.

This is a possibly complex but at the same time very elegantly written manuscript that clearly contributes to our understanding of the germination process and the interplay of GA and ABA during the germination process.

In my view, there are only two criticisms that have to be made to this paper:

First, the previously reported interaction of PIL/PIF transcription factors (such as the PIL5 protein studied in the manuscript) with DELLA repressors (2 Nature publications) and the corresponding functional implications are neither properly introduced nor included in the data discussion: As far as I can tell, only the Deng lab publication is mentioned (but neither properly introduced nor discussed), the Prat lab publication is neither mentioned nor discussed.

Second, although the genetic and physiological work is very detailed and convincing, no attempts have been made to delineate the underlying biochemical mechanisms and to directly link the proteins under investigation (e.g. DELLA - PIL5) at the cellular or biochemical level. The authors may want to argue in a rebuttal why this is not necessary.

Although the paper seems long all paragraphs are written in a concise manner.

Referee #3 (Remarks to the Author):

This paper explores the role of GA/DELLA protein and of ABA/ABI1 in the inhibition of seed germination by far-red light exposure. The model proposed suggests that: 1) DELLA proteins RGL2, GAI, and RGA are directly responsible for the stimulation of ABA synthesis and the expression of ABA-response factors AB13 and AB15.; 2) events leading to seed germination (testa rupture and endosperm rupture) are controlled independently by GA signaling and ABA signaling, respectively; and 3) gene transcription of DELLA proteins RGA and GAI is influenced by phytochrome B (phyB) and the bHLH transcription factor, PIL5, during a far-red light response. Overall, this is a interesting study that broadens our understanding of how ABA and GA regulate seed germination. The idea of examining whether ABA and GA act in different tissue types is clever. However, in some cases the data presented do not fully support the conclusions. There are also some problems with inadequate controls, insufficient descriptions of sample sizes and statistical significance, and incomplete figure legends. In particular, some of the protein blot analysis should be supported by RNA analysis. As a matter of opinion, I don't believe the authors should conclude that ABA alone is important for endosperm rupture since this is inconsistent with previously published work of Bethke et al. showing that the GA stimulates vacuolization in the aleurone/endosperm layer of Arabidopsis. I suppose you could make the argument that this might be an indirect effect of GA on ABA synthesis. This needs to be discussed in the Discussion section. There is much to think about here.

Introduction: The introduction provides a nice overview of both ABA and GA signaling models, the influence of light signaling on these pathways, and neatly outlines "key aspects" of all three that have yet to be tested. One suggestion is to reorganize how you address the role of SOMNUS, a bHLH protein that is believed to repress seed germination. It is mentioned briefly in the introduction but none of the experiments attempt to expand the understanding of this protein, and yet there are conclusions drawn in the discussion without any direct evidence. You may consider placing these hypotheses at the very end of the manuscript and point out that they are suggested directions for follow-up, or include the relevant data if you have it.

Results: This manuscript attempts to demonstrate the following: 1) under FR conditions, low GA levels allow an overaccumulation of GAI, RGA, and RGL2 which are responsible for blocking testa rupture; 2) the role of PIL5 influences RGA and GAI levels only under FR conditions where GA levels are not drastically reduced; 3) GAI and RGA can complement rgl2 mutants when under the control of the RGL2 promoter sequence. It is also suggested that: 1) DELLA-dependent blockage of testa rupture promotes an increase in ABA which prevents endosperm rupture; 2) ABA-dependent repression occurs through AB13. However, data indicating endogenous ABA levels have been measured, both in the controls, or the mutant lines, are never presented. Additionally, many of the

experiments attempting to characterize how accumulation of DELLA protein stimulates ABA endogenously are done in the presence of paclobutrazol to synthesize a "low GA environment." PAC is a P450 inhibitor that affects GA synthesis. More importantly in this case it can inhibit P450 CYP707A which is responsible for ABA catabolism, and may lead to an "artificial" increase in ABA pools. Without data representing the proper controls (endogenous ABA measurements and pil5/abi mutant lines crossed into a ga1-3 (GA biosynthesis mutant) background) it is not possible to determine whether the results reported are real or an artificial result caused by the treatment. PIL5's role is to influence GAI and RGA only under FR conditions where GA levels are not drastically reduced. Is this what was meant? If so how was the experimental condition imposed (methods description/reference) and was endogenous GA measured? Finally, in the methods and materials there is a list of the seed donors. However, it might be more useful to provide a table listing all of the mutants, backgrounds, and ecotypes. It is an easy way for the reader to identify what is being compared in each figure without having to flip through to the methods and read through the paragraph each time.

The results section starts off by stating some assumptions based on the published literature. The statement, "Previous reports have indicated that in conditions other than white light illumination, RGL2 is no longer the main DELLA factor repressing seed germination in response to low GA level (Cao et al 2005)" is far too strong given the data in the paper. At least change "indicated" to "suggested", please! In the Cao et al 2005 paper, the rgl2 mutation was the only DELLA mutant that showed any rescue of germination in suggesting that it is the major DELLA. Also, Cao et al 2005 measured % germination in the single, double, and triple DELLA mutants in ga1-3 after only 3.5 days of incubation at 22C. This is far too short an incubation period to consider this a final determination of the germination potential of these mutants in the dark OR light - at least 5d in the light, and sometimes 14 days are needed for germination in the dark. I think that Cao et al 2005 succeeded only in showing that the effect of DELLA mutants is additive.

Figure 1: This is an experiment designed to compare the role of RGL2, RGA, and GAI in far-red vs. white light perception as well as the roles that these proteins play in testa rupture. It is also designed to monitor what happens to AB15 protein accumulation during FR or WL responses. The photos in 1A and B illustrate germination or no germination using a photograph at 30h or 5d. Fig. 1D shows % testa rupture at 60h, but there is no indication of sample size, number of times the experiment was replicated, or error bars for these figures or in the figure legend. It is impossible to judge statistical significance. Also this is a short length of time at which to score germination suggesting that this might be a matter of germination rate rather than total germination potential. Did you make any observations at late time points that you could describe? In these photos where you show many seeds in a frame and say there was no testa rupture you are leaving too much to trust - no one can see the truth of the statement in this photo. And how do you know that there is no rupture of the aleurone/endosperm layer without dissecting it out? You must document that this result was quantitated by putting a number next to the photo or in the legend. It makes it hard to tell how many seeds aren't germinating. More importantly, the authors suggest that RGL2 is the primary DELLA responsible for blocking testa rupture in WL, whereas all three serve redundant functions in FR. The germination (%endosperm rupture) is better documented under the western analysis (1C). Fig. 1c supports the conclusion that ABI5 protein disappearance is associated with germination and that the rgl2 mutation alone is not sufficient to allow ABI5 disappearance in the dark. It is possible that RGA or GAI serve more distinct roles than previously thought. What happens in the rgl2 rga and rgl2 gai double mutants (just curiosity)?

Figure 2: This experiment shows that if you introduction of rgl2 gai rga into the ga1-3 background allows testa rupture (not germination) even in the ga1-3 background. This is associated with high levels of ABI5 protein suggesting that ABA signaling is blocking endosperm rupture not testa rupture. It would be nice to show the same time points in all figures looking at the ABI5 levels (12h-5 days). At the very least, show 3 and 5 d for all.

Figure3: This figure is a nice follow-up because it demonstrates that even in the presence of ABA, GA causes the disappearance of RGA, GAI, and RGL2 protein without causing AB15 disappearance (3A). This strengthens the argument for GA or ABA specific roles during germination. Mention that this is consistent with previous data of Zentella et al. This figure also goes on to show that in the presence of ABA (without GA in ga1-3) there is no testa rupture and presumable no germination but in the presence of both GA and ABA you see testa rupture without endosperm rupture (3B). The

Figure 3B legend fails to give the time point shown (day 3 or day 5?), and once again no sample size or standard deviation are given.

Figure 4: The purpose of this set of experiments is to demonstrate that when GAI and RGA are expressed under the control of the RGL2 promoter in the rgl2-13 mutant background under "low GA" conditions they are able to inhibit seed germination arguably by blocking testa rupture. There appears to be little to no germination in the pro-RGL2::GAI, ::RGA, or WT the images and % testa rupture numbers are given (good!), but sample size (n= X) should be given in the legend. Why is testa rupture measured at 54 hours instead of 5 days as in previous experiments? What happens at 3 days? Again, for consistency it might make sense to show all time points or provide a better explanation for these experimental conditions. It appears that RGL2p:GAI complements rgl2 better than RGL2p:: RGA, suggesting a stronger influence by GAI. This is consistent with higher accumulation of GAI in the protein blot in Figures 4B, 5B. The argument that RGL2 shows a stronger regulation of seed germination in white light compared to after FR treatment would be better supported if you showed that whereas the RGL2 promoter is expressed better than GAI and RGA in white light (cite Lee et al 2002 and Tyler et al 2004), GAI and RGA are expressed at higher levels than RGL2 following a pulse of FR light. RNA analysis is also need to show if the effects are in Figure 4B are transcriptional and not posttranscriptional. In Figure 4B you also need to show RGA and GAI protein accumulation in the rgl2 mutant in order to show the effect of the RGL2-promoter constructs in the correct background. Oddly, this is shown in 4C. This is needed to demonstrate the correlation between DELLA protein expression and AB15 accumulation (via PAC treatment). There is one caveat here in that PAC is known to increase endogenous ABA levels -so showing the difference in protein accumulation between different genotypes is important. You should make your rationale for dealing with the PAC-caveat clear in the text.

Figure 5: The rationale for this experiment is a bit hard to follow until one gets to figure 6A. PIL5 gene induces expression of RGA and GAI mRNA after FR. The point of figure 5AB is that mutations in pil5 allow testa rupture after FR only if GA synthesis is normal (no PAC). Figure 5B shows that pil5 causes a decrease (but not complete absence) of RGA and GAI protein in the absence of PAC following FR. But pil5 does not stop RGA and GAI protein accumulation when PAC is added. I think the interpretation that this is due to PAC blocking the GA-triggered degradation of DELLAs is correct. But you need to make clear that this is an only opinion (it could be wrong) since you do not show a corresponding analysis of RNA accumulation for RGA and GAI.

Figure 6: This experiment uses ABI5 protein accumulation as a reporter for ABA accumulation. This is not a great idea. In the imbibition experiments Piskurewicz 2008 Figure 7, it is clear that ABI5 levels decrease as endogenous ABA decreases but ABI5 does not increase when ABA levels rise again by 72h. I would be more comfortable if you refer to increased ABI5 as an indicator of increasing ABA signaling.
This experiment shows protein blot analysis of AB15 protein accumulation under the same conditions as Figure 5. In the absence of PAC AB15 accumulation in pil5 mutants is lower in WL than after FR treatment by 72 hours. The lower ABI5 levels correlated well with higher germination (Fig. 5). However, in the presence of PAC, AB15 levels were higher in both WT and pil5 mutants in either light treatment and correlated with failed seed germination. The authors' reasoning is that the higher ABI5 levels on PAC result from higher DELLA RGA and GAI levels, and suggests that the DELLAs are causing higher ABA accumulation/signaling. Endogenous ABA measurements to confirm would confirm this would be helpful. But you cold get just loosen the language a bit. I agree that you have demonstrated a change in ABA signaling.

Figure 7: In this experiment the authors are trying to demonstrate that ABA acts through AB13 to inhibit endosperm rupture in FR conditions. They compare a seed's ability to germinate in WT compared to pil5, aba1 (ABA biosynthetic mutant), and abi3 mutants. Seeds were imbibed under both WL and FR conditions in the presence of PAC. The goal of this experiment was to determine if endosperm rupture occurs in low ABA conditions as was predicted by their model. One noted observation was that aba1 seeds do not germinate because there is no testa rupture (due to accumulation of DELLA in the presence of PAC). With mechanical testa rupture it was noted that seeds are then able to germinate. Based on experience, I am amazed that you were able to puncture the testa without damage to the endosperm. Usually, one only manages to puncture one layer a certain percentage of the time. I respect you for doing this experiment, but how can you prove that the endosperm was not ruptured? Damage to the endosperm would stimulate seed germination on

PAC. In Figure 7 the "puncture" image only depicts a fraction of the total seeds imaged in the "no puncture lane". How many seeds total were punctured and of that total how many germinated? What fraction of the total number of seeds does this represent? Over what time frame are these seeds monitored for germination? There is no indication in the figure legend. In the image it appears that almost all of the abi3 seeds have germinated without puncture. However in the results discussion you mention that germination ratios are variable according to seed batch. The supplementary Fig. 3A give a good control showing viability/germination of punctured seeds (label them so in the figure). The pil5 looks composed or spliced together. This is OK, but then one must have numbers for germinated seeds per total seeds. A very important point is that abi3 shows germination even in the absence of puncture. There is a statement that there was variability in seed batches. Thank you for your honesty. Please explain why you think there is batch to batch difference?

The data do not support the conclusion on p. 15 that " observations strongly support... only ABA levels determine the potential of a seed to rupture its endosperm, irrespective of GA levels." Your own experiments show that testa rupture requires GA and that endosperm rupture occurs after testa rupture! You should just as emphatically state that GA is important for testa rupture in seed germination. Seed germination is classically defined as the emergence of the radicle - this requires both testa and endosperm rupture. You cannot redefine germination to be synonymous with endosperm rupture as on p. Previous

Figure 8: This experiment measures DELLA proteins RGA and GAI in aba1, abi3, and WT seeds under FR and PAC treatment. This figure is intended to show DELLA accumulation and further evidence for the lack of testa rupture as a physical block to endosperm rupture in Figure 7. The only suggestion for this figure is that it would be nice to see what RGL2 and AB15 protein levels look like as well. It would also be nice to see if protein levels persist throughout the 5 day time or after puncture.

Discussion: In the discussion the authors mention that the critical findings of this research are: 1) in the absence of GA synthesis RGA and GAI redundantly work with RGL2 to repress testa rupture and repress endosperm rupture by increasing endogenous ABA levels; 2) PIL5 dependent gene and protein expression of GAI and RGA. As mentioned above the investigators do not explore the effects of RGA or GAI alone, rather the comparison is always between rgl2 and rgl2/gai/rga.. Additionally, GAI appears to be the more prominent protein when comparing relative germination rates, and protein accumulation. Could it be that it plays a more distinct role in testa rupture than RGA? The other assertion here is that accumulation of these DELLAs leads to increases in endogenous ABA levels. However, for this study ABA levels are never measured or compared between lines that are treated or not treated with PAC. It is possible that PAC treatment does not induce a significant change in ABA pools but controls need to be established to make sure that increases in AB13 are due to increases in ABA pools that are modulated by DELLA and not PAC.

Methods and Material/Figure Legends: Overall the investigators should include more detail in there figures and figure legends (as indicated above). In addition to germination numbers it would also be nice to see % testa rupture as well. Are FR conditions of the authors own design? If not could a reference be provided?

Supplemental Figures: Overall these figures provide useful information to the reader but are lacking in some detail in figure legend. In Figure 1 % germination is useful. Figure 3A should be labeled as a puncture treatment. Also these images sometime look like they have been merged together form two or several separate images (3A pil5, and 4 puncture of abi3-9). How many were plated in each group and was there a fraction that didn't germinate?

Methods:

You refer to Piskurewicz for the anibodies used to recognize RGL2, GAI and RGA. Did that previous paper contain gai-t6 and rga-24 mutant controls to check the specificity of the antibody for these proteins? If not, please include these controls in the supplementary data using the same treatments shown in this paper to make sure we are looking at the right proteins. (Not a major point, but it is important to be sure of this.) Suppl. Fig 2 shows ga1-3 rgl2, but not ga1-3 rga-24 and ga1-3 gai-t6.

1st Revision - authors' response 21 May 2009

Answers to reviewer #1:

1) Although title of this manuscript include "DELLA-dependent enhancement of ABA synthesis", authors did not measure the level of ABA. Authors rely on ABI5 activity for endogeneous levels of ABA. Authors should measure endogenous ABA contents in WT, ga1, and ga1/rga/gai/rgl2 mutant lines as they did so for the previous report, especially authors are reporting different sets of mutants here. Also, it will be interesting to see if any of ABA biosynthetic genes are affected in the mutants tested.

Here we consider the role of DELLA-dependent enhancement of ABA synthesis in a context of low GA levels (i.e. in a ga1 background or in PAC-treated seeds). A time course of endogenous ABA contents in WT and ga1 seeds under far-red conditions was previously performed in Seo et al 2006 (Fig 1B, Set et al Plant Journal 48, 354-366). We have now compared ABA contents in ga1, ga1/rgl2 and ga1/rgl2/gai/rga plants and they are consistent with our claims. We added this data in the manuscript (Figure 2, see changes in text in bold). We monitored NCED6 mRNA levels and the results are consistent with the endogenous ABA levels measurements (suppl. Fig. 3). However, we argued in the manuscript that following the expression of metabolic gene expression does not discriminate whether a) occurrence or absence of seed germination is due to change in hormone metabolism or b) change in hormone metabolism reflects the commitment or its absence of a seed to germinate. Low endogenous ABA levels in ga1/rga/gai/rgl2 relative to ga1 further confirms the view that DELLA factors promote higher endogenous ABA levels, but the precise mechanisms it entails remain to be investigated.

2) Also in the title, authors argue that "DELLA proteins enhance ABI3 activity". But, no direct data to show the case a) Is ABI3 expression could be affected in della mutants? b) Can DELLA overexpression line enhance the expression of ABI3 gene and ABI3-mediated downstream target genes?

We provide a RNA blot analysis of ABI3 mRNA expression showing that ABI3 expression is up-regulated and maintained in ga1 but not in ga1/rgl2 and ga1/rga/gai/rgl2 under white light conditions (Figure 9B). ABI3 mRNA expression is up-regulated and maintained ga1 and ga1/rgl2 but not in ga1/rga/gai/rgl2 under far-red light conditions (Figure 9B). Finally, ABI3 expression is up-regulated and maintained in rgl2/pro-RGL2::GAI and rgl2/pro-RGL2::RGA but not in rgl2 under white light conditions in PAC-treated seeds (Figure 9C). ABI5 is an ABI3 downstream target gene (Lopez-Molina et al 2002) and it is up-regulated in pro-RGL2::GAI and pro-RGL2::RGA (Figure 5C).

3) Authors mentioned that DELLAs act upstream of ABIs (ABI3 and ABI5) through enhancement of ABA synthesis, to prove this hypothesis, Authors should measure the expression level of DELLA genes, ABI3 and ABI5 genes under -ABA and +ABA condition.

We are not sure we understand this point. It has been shown in previous studies how RGL2, GAI, RGA, ABI3 and ABI5 are expressed in presence and absence of ABA (Piskurewicz et al 2008, Perruc et al. 2007, Lopez-Molina et al 2001, Lopez-Molina et al 2002 etc..).

4) Authors only focused on inactive PHYB condition irradiated with far-red light for the seed germination assay. Even PHYB is the main photoreceptor regulating seed germination, PHYA photoreceptor is involved in seed germination as well (Kim et al., 2008; Plant Cell and etc.). Prolonged far-red light irradiation could stabilize PHYA photoreceptor and activated PHYA can also induce the seed germination. Thus authors should do the PHYA-mediated seed germination test. It will be useful to gain the insight on overall relationship between DELLA proteins and Phytochromes in the seed germination.

It is unlikely that the standard far-red assay used in this study activates PHYA significantly since it fully inhibits WT seed germination germination, as shown in supplementary figure 1.

Having said this, we are respectfully uncertain that we fully understand the point this reviewer is trying to raise. During germination, PHYA has been described as an activator of seed germination

under specific conditions of Far-red light illumination when PHYB is already inactive (i.e. a 4-hour pulse of far-red light activates PHYA during the dark period with PHYB previously inactivated by a 5 min far-red pulse). Our manuscript is only concerned with far red-dependent repression of seed germination, focusing on the inhibitory role of DELLA factors, ABA and ABI factors with or without GA synthesis. All along the manuscript we have assumed that our assay blocks germination mainly because PHYB is inactive. Nevertheless, this is not a key assumption and we do not rule out that some other phytochrome-dependent repression of seed germination is taking place in our assay. Our assumption is based on the conclusion of numerous genetic studies that have established that an early pulse of far-red light (as used in our experimental set-up) blocks germination in a PHYB-dependent manner.

We might perceive the suggestions of this referee ("to gain insight on the overall relationship between DELLA proteins and phytochromes"; "authors should do the PHYA-mediated seed germination test") as to inquire how active phytochromes stimulate germination and the role of DELLA factors in this context. This issue is of great interest but very general and beyond the scope of this work, at least as presented by this reviewer when he suggests to perform a PHYA-mediated seed germination test. Indeed, both PHYB and PHYA can stimulate germination upon red and far-red irradiation, respectively. Therefore, to gain insight on the overall relationship between DELLA proteins and phytochromes would require a side-by-side study that compares PHYB-dependent stimulation of seed germination under red light versus PHYA-dependent stimulation of seed germination under far-red light. This is a challenging topic if only one considers the question of how phytochromes stimulate GA synthesis, which is still unresolved, as correctly noted by this reviewer (see his point 6). Comparing PHYB-dependent and PHYA-dependent stimulation of seed germination is further complicated by the fact that in each case light treatments are performed at different times upon imbibition so that their effects on GA- and ABA-response factors or effects on hormone metabolism are not readily interpretable without further experimental inquiry.

5) Although XERICO is previously reported as a target of GAI and RGA, there is no experiment on XERICO in the seed germination condition in this manuscript.

We performed the experiment (new suppl. Fig 4). The pattern of XERICO expression is consistent with the notion that it is a DELLA-target gene and that it may participate to elevate endogenous ABA levels

6) A model proposed in Fig.9 shows that PHYs can regulate GA directly and that PIL5 only regulates RGA and GAI. It is not consistent with other reports. There is no evidence on PHYs can directly regulate GA synthesis (even in canopy condition). It has been reported that PIL5 can regulate GA biosynthetic genes, as authors indicated in the text.

We have modified the model (dashed arrow instead of solid arrow) and the legend (bold text) to ensure to convey the notion that phytochromes may indirectly regulate GA synthesis.

It should be pointed out that the group of Choi did not state that PIL5 regulates directly GA biosynthetic genes and that it remains an open issue how it does so. Concerning PIL5, we wished to emphasize two points in the discussion regarding PIL5ís role on regulating metabolic gene expression: 1) "..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) (Kim et al, 2008; Oh et al, 2007)" and 2) "Without further investigation, a given state of metabolic gene expression or hormone levels [as in pil5 mutants] may be interpreted as the consequence rather than the cause of seed germination." Based on these two arguments, we feel it remains unclear whether PIL5 indeed regulates GA biosynthetic genes. Nevertheless, since we cannot exclude it, we modified the model accordingly (two inhibitory arrows between PIL5 and GA and legend modification in bold).

Minors
All germination assays should include the WT control.

OK

In INTRODUCTION part, page 5. SOM is not bHLH protein, but a C3H zinc finger protein

OK

Answers to reviewer #2:

In my view, there are only two criticisms that have to be made to this paper:

First, the previously reported interaction of PIL/PIF transcription factors (such as the PIL5 protein studied in the manuscript) with DELLA repressors (2 Nature publications) and the corresponding functional implications are neither properly introduced nor included in the data discussion: As far as I can tell, only the Deng lab publication is mentioned (but neither properly introduced nor discussed), the Prat lab publication is neither mentioned nor discussed. Second, although the genetic and physiological work is very detailed and convincing, no attempts have been made to delineate the underlying biochemical mechanisms and to directly link the proteins under investigation (e.g. DELLA - PIL5) at the cellular or biochemical level. The authors may want to argue in a rebuttal why this is not necessary.

We provide below a paragraph that could possibly included at the end of the discussion and that discusses the matters raised by this reviewer. We would be glad to include any additional interesting point we might have forgotten or not aware of. We leave it to the reviewer and the editor whether we should expand further our discussion by incorporating the paragraph.
Our personal opinion however is that that the discussion is already quite long and the points discussed in the paragraph do not provide any substantial thinking that has not been already discussed elsewhere (see for instance Zentella et al 2007. Piszkurewicz et al 2008). Adding the paragraph below would exceed the total character count allowed. Our primary goal for the discussion was to focus on the respective role of GA and ABA on the control of the early seed germination processes of testa and endosperm rupture, a topic that is, we think, often confusedly treated in the literature.

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 understanding 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 (de Lucas et al. 2008, Feng 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 in order 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 that 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 plays a central and temporally restricted regulatory role (Lopez-Molina et al 2001, Piskurewicz et al 2008). The regulation of hypocotyl elongation occurs well after the developmental window where 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.

Answers to reviewer #3:

However, in some cases the data presented do not fully support the conclusions. There are also some problems with inadequate controls, insufficient descriptions of sample sizes and statistical significance, and incomplete figure legends. In particular, some of the protein blot analysis should be supported by RNA analysis.

These issues are considered below.

As a matter of opinion, I don't believe the authors should conclude that ABA alone is important for endosperm rupture since this is inconsistent with previously published work of Bethke et al. showing that the GA stimulates vacuolization in the aleurone/endosperm layer of Arabidopsis. I suppose you could make the argument that this might be an indirect effect of GA on ABA synthesis. This needs to be discussed in the Discussion section. There is much to think about here.

In Piskurewicz et al. 2008, we pointed out in the discussion that the molecular mechanisms underlying endosperm rupture are not fully understood. Here, as in Piskurewicz et al. 2008, we refer to endosperm rupture only when the radicle visibly protrudes out of the seed coat. This has the advantage of ensuring reliability and quantification. In this sense, our experiments indeed indicate that ABA alone is important for endosperm rupture. We emphasized this point again in the discussion ("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)."

One suggestion is to reorganize how you address the role of SOMNUS, a bHLH protein that is believed to repress seed germination. It is mentioned briefly in the introduction but none of the experiments attempt to expand the understanding of this protein, and yet there are conclusions drawn in the discussion without any direct evidence. You may consider placing these hypotheses at the very end of the manuscript and point out that they are suggested directions for follow-up, or include the relevant data if you have it.

We do not identify which conclusions we would have drawn in the discussion, as commented by this reviewer. We just discuss and speculate about our results in the light of the conclusions drawn by the group of Choi based on their work performed with SOMNUS. Our manuscript does not make any substantial statement about SOMNUS.

It is also suggested that: 1) DELLA-dependent blockage of testa rupture promotes an increase in ABA which prevents endosperm rupture;

Please see model in figure 10. We claim that DELLA factors 1) block testa rupture and 2) promote endogenous ABA synthesis. We have not claimed that the effect of blocking testa rupture is what promotes an increase in endogenous ABA. Both processes (testa rupture and increased ABA levels) are treated separately.

However, data indicating endogenous ABA levels have been measured, both in the controls, or the mutant lines, are never presented.

We are providing endogenous ABA measurements in the revised version of the manuscript (see new Figure 2).

Additionally, many of the experiments attempting to characterize how accumulation of DELLA protein stimulates ABA endogenously are done in the presence of paclobutrazol to synthesize a "low GA environment." PAC is a P450 inhibitor that affects GA synthesis. More importantly in this case it can inhibit P450 CYP707A, which is responsible for ABA catabolism, and may lead to an "artificial" increase in ABA pools. Without data representing the proper controls (endogenous ABA measurements and pil5/abi mutant lines crossed into a ga1-3 (GA biosynthesis mutant) background) it is not possible to determine whether the results reported are real or an artificial result caused by the treatment.

This study is mainly concerned in showing that the emerging notion that DELLA factors block germination because they promote an elevation of endogenous ABA can be extended to the context of light-dependent inhibition of seed germination. In this sense, our studies on PIL5 are rather a secondary aspect of this manuscript. Nevertheless, we strongly believe that our experimental approach is valid (i.e. using PAC with pil5 mutants, see below) and that our conclusions would be of great interest to the readers because they situate the function of PIL5, an important regulator of seed germination, in the context of the main notion above that we wish to introduce. pil5/ga1 seeds are not immediately available to us. Generating pil5/ ga1 (and pil5/ga1/abi) seeds would take several months, perhaps a year. This would represent, we feel, an unnecessary delay for publication of this manuscript since it would not change its conclusion. It should be pointed out that ABA levels in pil5 and in ga1/pl5 mutant under FR conditions have been already reported by Oh et al, 2007. Consistently with our data, pil5 mutant had low ABA levels under FR conditions while in ga1/pil5 double mutant ABA levels raised to levels comparable to FR- treated WT seeds (Oh et al, 2007). We refer to those data in results section, page 13:
"pil5 mutants can germinate under FR conditions and this is associated with lower endogenous ABA levels. However, pil5 mutants cannot germinate in absence of GA synthesis (as in a ga1 background), which is associated with increased endogenous ABA levels (Oh et al, 2006), Oh et al, 2007). ".

We are fully aware that Unicazol or, to a much lesser extent, paclobutrazol (PAC) can inhibit P450 CYP707A, as previously reported (Saito et al Biosc. Biotechnol. Biochem. 70 (7), 1731-1739). This is why we carefully chose the inhibitor and its concentration. We previously showed that this does not lead to artificial results (Piskurewicz et al 2008). A number of additional reasons and results can be invoked to further support this claim. We summarize all of them here:

1) PAC-treated WT seeds do not germinate and accumulate high ABI5 protein levels in a manner indistinguishable from ga1 seeds (Piskurewicz et a. 2008). Adding GA to PAC-treated WT seeds (PAC+GA) triggers seed germination and a drop in ABI5 protein levels, in a manner indistinguishable from GA-treated ga1 seeds. These observations are consistent with the notion that the observed effects of PAC are the consequence of lower GA levels rather than the consequence of PAC-dependent inhibition of CYP707 activity.

2) The experiment described in 1) was performed under white light conditions (Piskurewicz et a. 2008). We repeated this experiment under far-red conditions leading to identical conclusions (suppl fig 10).

3) The observed elevation in endogenous ABA levels in PAC-treated seeds occurs beyond 24 hours after seed imbibition and is not observed in PAC-treated rgl2 seeds (Piskurewicz et a. 2008). This again suggests that higher endogenous ABA levels in PAC-treated seeds are due to high RGL2 protein, which is the consequence of low endogenous GA levels. [Note: ABA levels are high in dry seeds (50ng per gram of dry seeds) and they drop 10 fold within the first 12 hours upon normal imbibition (Piskurewicz et al. 2008). This drop most likely involves the activity of CYP707 hydroxylases (Okamoto et al Plant Physiol. 2006 Vol 141 pp 97-107)). We showed that PAC-treatment did not alter this massive drop in endogenous ABA levels, further consistent with the notion that PAC does not significantly inhibit CYP707 activity under our experimental conditions].

4) We performed a time course of endogenous ABA levels in ga1, which accumulate high ABI5 protein as in PAC-treated WT seeds, and in ga1/rgl2 seeds, which accumulate low ABI5 protein as in PAC-treated rgl2 seeds (Figure 2). As expected, the results are most similar to those obtained with PAC-treated seeds (Figure 2; Piskurewicz et al 2008). This further strengthens the validity of our experimental assays that use PAC.

5) As stated in the Piszkurewicz et al 2008, ga1 dry seeds accumulate higher endogenous ABA levels than WT and cannot germinate. ga1/aba1 mutant seeds are able to germinate (Leon-Kloosterziel et al. 1996, Plant J 10, 655-661). Moreover, treating ga1 seeds as well as PAC-treated WT seeds with norflurazon, an inhibitor of ABA synthesis, triggers their germination and is associated with a drop in ABI5 protein levels (see suppl. Fig. 8A and 8B in Piszkurewicz et al 2008).

Taken together, these observations provide independent support that our observations using PAC

result from lower GA levels leading to an elevation in endogenous ABA levels rather than artefactual effects. These arguments (point 1 to 5) are presented in suppl fig 10. We refer to this potential caveat and to suppl. Fig. 10 in the legend of the figures where PAC is used.

PIL5's role is to influence GAI and RGA only under FR conditions where GA levels are not drastically reduced. Is this what was meant? If so how was the experimental condition imposed (methods description/reference) and was endogenous GA measured?

Yes this is what is meant. We used PAC-treated pil5 mutants. Endogenous GA was not measured (see discussion above about the validity of using PAC lower GA levels in seeds)

Finally, in the methods and materials there is a list of the seed donors. However, it might be more useful to provide a table listing all of the mutants, backgrounds, and ecotypes. It is an easy way for the reader to identify what is being compared in each figure without having to flip through to the methods and read through the paragraph each time.

OK, this is now suppl. Fig 11

The results section starts off by stating some assumptions based on the published literature. The statement, "Previous reports have indicated that in conditions other than white light illumination, RGL2 is no longer the main DELLA factor repressing seed germination in response to low GA level (Cao et al 2005)" is far too strong given the data in the paper. At least change "indicated" to "suggested", please!

Ok, we changed the wording of this sentence (change is in bold)

Figure 1: Fig. 1D shows % testa rupture at 60h, but there is no indication of sample size, number of times the experiment was replicated, or error bars for these figures or in the figure legend. It is impossible to judge statistical significance. Also this is a short length of time at which to score germination suggesting that this might be a matter of germination rate rather than total germination potential. Did you make any observations at late time points that you could describe? In these photos where you show many seeds in a frame and say there was no testa rupture you are leaving too much to trust ñ no one can see the truth of the statement in this photo. And how do you know that there is no rupture of the aleurone/endosperm layer without dissecting it out? You must document that this result was quantitated by putting a number next to the photo or in the legend. It makes it hard to tell how many seeds aren't germinating.

For each seed batch a minimum of two replication experiments was performed (this information is now added in material and methods). In the material and methods section we stated that "A minimum of three independently grown seed batches were used for measuring percent of testa and endosperm rupture events". We also stated "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.". We used the same approach in Piskurewicz et al 2008.

In figure 1, all seed batches behaved similarly: at 60h we scored testa rupture (not endosperm rupture) and under white light, there is 100% testa rupture in ga1/rgl2 and ga1/rgl2/gai/rga and 0% in ga1 (Fig 1B). At 60h, under far-red light conditions, there is 100% testa rupture in ga1/rgl2/gai/rga and 0% in ga1/rgl2 and ga1 (Fig 1B). These are highly reproducible and statistically significant results (percentage values that are either 0% or 100% with standard deviation equal to zero) with different seed batches. Testa and endosperm rupture events are measured using high-resolution images of seed populations. Suppl. Fig1 in Piskurewicz et al. shows the typical aspect of a seed in such images. We can provide digital images if needed. We did not quantify testa rupture events beyond 60 hr in ga1 (white light) or ga1/rgl2 (white light and far-red) because they remain extremely rare, even after 5 days. We modified the legend to include the sample size (between 150 and 300 seeds) and number of seed batches used for each particular experiment ("(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)

Concerning the percentage numbers for endosperm rupture events shown in Fig 3A, the numbers correspond to the actual percentage in the same seed material used for the western blot shown in Fig 3B. As for testa rupture the numbers for endosperm rupture are eloquent: at 96h (or 5 days) endosperm rupture is either 0% or 100% depending on the genotype. So here again these are highly statistically significant results (legend is also modified: "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).").

-> Figure 1A is meant to illustrate the typical appearance of seeds (legend is also modified in this sense) rather than making a quantitative statement.

-> As stated above, we did not dissect the aleurone/endosperm as we only consider endosperm rupture events as defined and described above.

What happens in the rgl2 rga and rgl2 gai double mutants (just curiosity)?

We do not have rgl2/rga or rgl2/gai double mutant seeds. Sorry. However, based on the work of Cao et al (Cao et al 2005, Planta 223(1): 105-113), which showed poor germination in ga1/rgl2/rga and ga1/rgl2/gai double mutant seeds, we would anticipate that they retain high ABA and high ABA levels under far-red light conditions. We think, based on the research of Cao et al, that RGA and GAI are indeed redundant.

Figure 2: It would be nice to show the same time points in all figures looking at the ABI5 levels (12h-5 days). At the very least, show 3 and 5 d for all.

Keep in mind that ga1/gai/rga/rgl2 plants give a very low seed production yield and that it is not always possible or easy to perform a complete time course. All figures now have at least the time points 3 and 5 days.

Figure3: This figure is a nice follow-up because it demonstrates that even in the presence of ABA, GA causes the disappearance of RGA, GAI, and RGL2 protein without causing AB15 disappearance (3A). This strengthens the argument for GA or ABA specific roles during germination. Mention that this is consistent with previous data of Zentella et al.

OK, done.

The Figure 3B legend fails to give the time point shown (day 3 or day 5?), and once again no sample size or standard deviation are given.

Figure legend has been changed ("three independent seed batches (n=150-200), testa or endosperm rupture percentage is always either 0% or 100% depending on the treatment and the light condition as shown in the histogram").

Figure 4: The purpose of this set of experiments is to demonstrate that when GAI and RGA are expressed under the control of the RGL2 promoter in the rgl2-13 mutant background under "low GA" conditions they are able to inhibit seed germination arguably by blocking testa rupture.

Not exactly. We rather want to suggest that germination is prevented as a result of both the repression of testa and endosperm rupture. What is crucial however is the repression of endosperm rupture which is due to high ABI5 protein levels in rgl2/pro-RGL2::GAI and rgl2/pro-RGL2::RGA. Remember that PAC-treated abi5 mutants germinate (i.e. rupture endosperm) without prior visible rupture of the testa, unlike PAC-treated rgl2 seeds.

There appears to be little to no germination in the pro-RGL2::GAI, ::RGA, or WT the images and % testa rupture numbers are given (good!), but sample size (n= X) should be given in the legend. Why is testa rupture measured at 54 hours instead of 5 days as in previous experiments? What happens at 3 days? Again, for consistency it might make sense to show all time points or provide a better explanation for these experimental conditions.

We have now included the average testa rupture percentage at 96h (n=2). We also added an histogram showing testa and endosperm rupture percentages at 96h (n=2). See suppl. Fig 5B. Fig 5B now includes the percentage of endosperm rupture for each time point for that particular seed material.

It appears that RGL2p:GAI complements rgl2 better than RGL2p:: RGA, suggesting a stronger influence by GAI. This is consistent with higher accumulation of GAI in the protein blot in Figures 4B, 5B. The argument that RGL2 shows a stronger regulation of seed germination in white light compared to after FR treatment would be better supported if you showed that whereas the RGL2 promoter is expressed better than GAI and RGA in white light (cite Lee et al 2002 and Tyler et al 2004), GAI and RGA are expressed at higher levels than RGL2 following a pulse of FR light.

The arguments leading to the conclusion that RGL2 is the main DELLA factor under white light conditions are based on considerations concerning relative (not absolute) mRNA and protein levels of RGL2 versus those of GAI and RGA. See Piskurewicz et al 2008.

It was previously shown that GAI and RGA mRNA expression is more stimulated by far-red light relative to that of RGL2 (Oh et al. 2007 Plant Cell 16(11): 3045-3058). In Piskurewicz et al. 2008 we showed that RGL2 mRNA expression increases relative to that of GAI and RGA in PAC-treated seeds under white light conditions.

RNA analysis is also need to show if the effects are in Figure 4B are transcriptional and not posttranscriptional.

OK done (suppl. Fig 5).

In Figure 4B you also need to show RGA and GAI protein accumulation in the rgl2 mutant in order to show the effect of the RGL2-promoter constructs in the correct background.

OK done (Figure 5B)

There is one caveat here in that PAC is known to increase endogenous ABA levels -so showing the difference in protein accumulation between different genotypes is important. You should make your rationale for dealing with the PAC-caveat clear in the text.

This issue has already been discussed above

Figure 5: The rationale for this experiment is a bit hard to follow until one gets to figure 6A. PIL5 gene induces expression of RGA and GAI mRNA after FR. The point of figure 5AB is that mutations in pil5 allow testa rupture after FR only if GA synthesis is normal (no PAC). Figure 5B shows that pil5 causes a decrease (but not complete absence) of RGA and GAI protein in the absence of PAC following FR. But pil5 does not stop RGA and GAI protein accumulation when PAC is added. I think the interpretation that this is due to PAC blocking the GA-triggered degradation of DELLAs is correct. But you need to make clear that this is an only opinion (it could be wrong) since you do not show a corresponding analysis of RNA accumulation for RGA and GAI.

OK, we changed the text as follows : "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."

Figure 6: This experiment uses ABI5 protein accumulation as a reporter for ABA accumulation. This is not a great idea. In the imbibition experiments Piskurewicz 2008 Figure 7, it is clear that ABI5 levels decrease as endogenous ABA decreases but ABI5 does not increase when ABA levels rise again by 72h. I would be more comfortable if you refer to increased ABI5 as an indicator of increasing ABA signaling.

We respectfully disagree. All the experimental evidence so far indicates that ABI5 is an excellent marker to follow endogenous ABA levels as long as the plant is within the developmental window where it responds to ABA (LLM 2001, discussed in Piskurewicz et al 2008). The counter-example that this reviewer refers to is not valid in this respect (Figure 7, left panel Piskurewicz et al 2008)

because the rise in endogenous ABA levels occurs between 48h and 72h upon normal imbibition. This is beyond the developmental time window where the plants can respond to ABA and arrest their growth by notably inducing ABI5. Beyond 48h upon imbibition, under normal conditions, the plant is rapidly growing, is committed to its vegetative state and the rise in endogenous ABA is part of the normal development of the plant (discussed in Piskurewicz et al 2008) but it no longer serves the function of inducing embryonic gene expression such as that of ABI5 (see also Lopez-Molina et al 2002). The same figure 7 (right panel) shows an exquisite correlation between ABI5 protein levels and endogenous ABA levels. Note that levels can be directly compared since the same amount of seed material is used in each lane (100 seeds) in the protein blot and endogenous ABA levels are per gram of initial dry seed material.

This experiment shows protein blot analysis of AB15 protein accumulation under the same conditions as Figure 5. In the absence of PAC AB15 accumulation in pil5 mutants is lower in WL than after FR treatment by 72 hours. The lower ABI5 levels correlated well with higher germination (Fig. 5). However, in the presence of PAC, AB15 levels were higher in both WT and pil5 mutants in either light treatment and correlated with failed seed germination. The authors' reasoning is that the higher ABI5 levels on PAC result from higher DELLA RGA and GAI levels, and suggests that the DELLAs are causing higher ABA accumulation/signaling. Endogenous ABA measurements to confirm would confirm this would be helpful. But you cold get just loosen the language a bit. I agree that you have demonstrated a change in ABA signaling.

We have now included endogenous ABA measurements (New Figure 2)

Figure 7: In this experiment the authors are trying to demonstrate that ABA acts through AB13 to inhibit endosperm rupture in FR conditions. They compare a seed's ability to germinate in WT compared to pil5, aba1 (ABA biosynthetic mutant), and abi3 mutants. Seeds were imbibed under both WL and FR conditions in the presence of PAC. The goal of this experiment was to determine if endosperm rupture occurs in low ABA conditions as was predicted by their model. One noted observation was that aba1 seeds do not germinate because there is no testa rupture (due to accumulation of DELLA in the presence of PAC). With mechanical testa rupture it was noted that seeds are then able to germinate. Based on experience, I am amazed that you were able to puncture the testa without damage to the endosperm. Usually, one only manages to puncture one layer a certain percentage of the time. I respect you for doing this experiment, but how can you prove that the endosperm was not ruptured?

We do not claim that we have not damaged the endosperm since we do not have precise means to assess it. Our criteria of observation here is to assess whether radicle elongation takes place since we cannot ascertain that endosperm is not damaged. The observations are consistent with our model: punctured PAC-treated pil5 mutants (which have high DELLA levels) do not elongate the radicle (consistent with high ABA and ABI levels) unlike punctured aba1 and abi3 seeds. We have improved the text to better convey these notions:

Results section:
"Thus, we reasoned that failure to rupture testa may also mask endosperm rupture events (i.e. defined here as visible radicle protusion out of the seed coat) 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, suppl. Fig. 7)."

Discussion:
"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 since puncture experiments rupture both testa and endosperm)."

In Figure 7 the "puncture" image only depicts a fraction of the total seeds imaged in the "no puncture lane". How many seeds total were punctured and of that total how many germinated? What fraction of the total number of seeds does this represent? Over what time frame are these seeds monitored for germination? There is no indication in the figure legend.

We un-voluntary forgot to include the numbers concerning the puncture experiments in the manuscript. We apologize. These experiments require technical skill since they be must be

performed rapidly under white light illumination upon seed imbibition (as detailed in material and methods) so as to ensure that the inhibitory effect of far-red light illumination on seed germination is maintained. We added the following in Materials and Methods: "In a typical experiment, 15-19 seeds are punctured under white light illumination under the stereomicroscope. The procedure takes between 5 and 10 minutes. Thereafter, 10-14 seeds are subject to far-red light irradiation whereas 5 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) (suppl Fig 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 suppl Fig 7."

In the image it appears that almost all of the abi3 seeds have germinated without puncture. However in the results discussion you mention that germination ratios are variable according to seed batch. The supplementary Fig. 3A give a good control showing viability/germination of punctured seeds (label them so in the figure). The pil5 looks composed or spliced together. This is OK, but then one must have numbers for germinated seeds per total seeds. A very important point is that abi3 shows germination even in the absence of puncture. There is a statement that there was variability in seed batches. Thank you for your honesty. Please explain why you think there is batch to batch difference?

The particular pil5 picture is composed because after puncturing seeds in this particular experiment, the 10 seeds were inadvertently spread out quite far apart from each other in the plate. Thus, they would have appeared rather small due to the lower magnification needed to accommodate them in a single picture. We therefore compiled photos to keep a pleasing seed magnification.

We previously showed that PAC-treated aba1 (as well as abi5 seeds) can "germinate", i.e. radicle protrusion without prior visible rupture of the testa (Piskurewicz et al. 2008). Thus, we were at first surprised that PAC-treated aba1 seeds did not germinate well in this manner under far-red light. However, as discussed in the manuscript, we reasoned that under white light, it is mainly RGL2 that is exerting an inhibitory effect on testa rupture. Under far-red light, RGA and GAI contribute further to repress testa rupture. This may explain why PAC-treated aba1 seeds batches germinate poorly under far-red (about 20%) since the mechanical pressure of the unruptured testa cannot be overcome by the radicle. In the case of abi3 mutants we indeed observed variability dependent on the allele or seed batch. [The particular batch shown in figure 8 germinates with a percentage of 85% (see table suppl. Fig 7)]. This could be due to the particular mechanical state of the testa in the particular ecotype (Ler or Col), the particular seed batch, the influence of the particular abi3 allele (ABI3 is a key regulator of seed maturation) or any combination of these factors.

The data do not support the conclusion on p. 15 that " observations strongly support... only ABA levels determine the potential of a seed to rupture its endosperm, irrespective of GA levels." Your own experiments show that testa rupture requires GA and that endosperm rupture occurs after testa rupture! You should just as emphatically state that GA is important for testa rupture in seed germination. Seed germination is classically defined as the emergence of the radicle - this requires both testa and endosperm rupture. You cannot redefine germination to be synonymous with endosperm rupture as on p. Previous

We changed the statement to :" 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 as visible radicle protusion out of the seed coat), irrespective of GA levels.

We do not pretend to redefine germination or underscore the role of GA but rather to attempt to contribute to better understand how the control of seed germination is regulated by GA and ABA. In the introduction we state: "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. " In the beginning of the discussion we state: "The salient findings of this study are: 1) in absence of GA synthesis, RGA, GAI and RGL2 redundantly act to a) repress testa rupture and b) repress endosperm rupture by increasing endogenous ABA levels, which in turn inhibit endosperm rupture through ABI3"

Figure 8: This experiment measures DELLA proteins RGA and GAI in aba1, abi3, and WT seeds under FR and PAC treatment. This figure is intended to show DELLA accumulation and further evidence for the lack of testa rupture as a physical block to endosperm rupture in Figure 7. The only suggestion for this figure is that it would be nice to see what RGL2 and AB15 protein levels look like as well. It would also be nice to see if protein levels persist throughout the 5 day timeÖ.

OK, done. See Figure 9A and new suppl Fig 8.

Ö or after puncture.

This particular proposed experiment is a genuine heroic puncturing endeavor indeed! Protein blot analysis requires dozen of seeds for a single time point. However, it should be noted that when non-dormant, sly1 seeds germinate despite high RGA, GAI and RGL2 protein accumulation (but low ABI5 accumulation)(Piszkurewicz et al 2008). We would therefore expect DELLA factors remain high but not ABI5.

Discussion: In the discussion the authors mention that the critical findings of this research are: 1) in the absence of GA synthesis RGA and GAI redundantly work with RGL2 to repress testa rupture and repress endosperm rupture by increasing endogenous ABA levels; 2) PIL5 dependent gene and protein expression of GAI and RGA. As mentioned above the investigators do not explore the effects of RGA or GAI alone, rather the comparison is always between rgl2 and rgl2/gai/rga.. Additionally, GAI appears to be the more prominent protein when comparing relative germination rates, and protein accumulation. Could it be that it plays a more distinct role in testa rupture than RGA?

We removed the word "redundantly" when summarizing the critical findings. However, based on the work of Cao et al we feel it is most likely that GAI and RGA act redundantly.

The other assertion here is that accumulation of these DELLAs leads to increases in endogenous ABA levels. However, for this study ABA levels are never measured or compared between lines that are treated or not treated with PAC. It is possible that PAC treatment does not induce a significant change in ABA pools but controls need to be established to make sure that increases in AB13 are due to increases in ABA pools that are modulated by DELLA and not PAC.

ABA levels are now measured (see Fig 2).

Methods and Material/Figure Legends: Overall the investigators should include more detail in there figures and figure legends (as indicated above). In addition to germination numbers it would also be nice to see % testa rupture as well.

OK, see our comments above.

Are FR conditions of the authors own design? If not could a reference be provided?

See material and methods

Supplemental Figures: Overall these figures provide useful information to the reader but are lacking in some detail in figure legend. In Figure 1 % germination is useful. Figure 3A should be labeled as a puncture treatment. Also these images sometime look like they have been merged together form two or several separate images (3A pil5, and 4 puncture of abi3-9). How many were plated in each group and was there a fraction that didn't germinate?

OK, see our comments above.

Methods: You refer to Piskurewicz for the anibodies used to recognize RGL2, GAI and RGA. Did

that previous paper contain gai-t6 and rga-24 mutant controls to check the specificity of the antibody for these proteins? If not, please include these controls in the supplementary data using the same treatments shown in this paper to make sure we are looking at the right proteins.

Antibody specificity was shown in supplementary Fig 15 of Piskurewicz et al. 2008.

1) Figure 1: Fig. 1D shows % testa rupture at 60h, but there is no indication of sample size, number of times the experiment was replicated, or error bars for these figures or in the figure legend. It is impossible to judge statistical significance. Also this is a short length of time at which to score germination suggesting that this might be a matter of germination rate rather than total germination potential. Did you make any observations at late time points that you could describe? In these photos where you show many seeds in a frame and say there was no testa rupture you are leaving too much to trust ñ no one can see the truth of the statement in this photo. And how do you know that there is no rupture of the aleurone/endosperm layer without dissecting it out? You must document that this result was quantitated by putting a number next to the photo or in the legend. It makes it hard to tell how many seeds aren't germinating.

Histogram in this figure shows now percentage of testa rupture events 5 days after imbibition.

2) The Figure 3B legend fails to give the time point shown (day 3 or day 5?), and once again no sample size or standard deviation are given.

Histogram in this figure shows percentage of testa and endosperm rupture events 5 days after imbibition.

3) This experiment shows protein blot analysis of AB15 protein accumulation under the same conditions as Figure 5. In the absence of PAC AB15 accumulation in pil5 mutants is lower in WL than after FR treatment by 72 hours. The lower ABI5 levels correlated well with higher germination (Fig. 5). However, in the presence of PAC, AB15 levels were higher in both WT and pil5 mutants in either light treatment and correlated with failed seed germination. The authors' reasoning is that the higher ABI5 levels on PAC result from higher DELLA RGA and GAI levels, and suggests that the DELLAs are causing higher ABA accumulation/signaling. Endogenous ABA measurements to confirm would confirm this would be helpful. But you cold get just loosen the language a bit. I agree that you have demonstrated a change in ABA signaling.

ABA levels in pil5 and in ga1/pl5 mutant under FR conditions have been already reported by Oh et al, 2007. Consistently with our data, pil5 mutant had low ABA levels under FR conditions while in ga1/pil5 double mutant ABA levels raised to levels comparable to FR- treated WT seeds (Oh et al, 2007). We refer to those data in results section, page 13:

"pil5 mutants can germinate under FR conditions and this is associated with lower endogenous ABA levels. However, pil5 mutants cannot germinate in absence of GA synthesis (as in a ga1 background), which is associated with increased endogenous ABA levels (Oh et al, 2006), Oh et al, 2007). "

4) Discussion: In the discussion the authors mention that the critical findings of this research are: 1) in the absence of GA synthesis RGA and GAI redundantly work with RGL2 to repress testa rupture and repress endosperm rupture by increasing endogenous ABA levels; 2) PIL5 dependent gene and protein expression of GAI and RGA. As mentioned above the investigators do not explore the effects of RGA or GAI alone, rather the comparison is always between rgl2 and rgl2/gai/rga.. Additionally, GAI appears to be the more prominent protein when comparing relative germination rates, and protein accumulation. Could it be that it plays a more distinct role in testa rupture than RGA?

The antibody we use to detect GAI and RGA proteins was raised against GAI antigen. This might in part explain why GAI appears more prominent protein when compared to RGA. It might be due to higher antibody affinity towards GAI.

