

## Article Addendum

# The GA-signaling repressor RGL3 represses testa rupture in response to changes in GA and ABA levels

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We recently reported that the DELLA factor RGL2 represses testa rupture in response to changes in ABA and GA levels. Here, we provide genetic evidence that this observation extends to RGL3, another DELLA factor whose function was not previously characterized. However, RGL3's repressive activity was seen only in an *rgl2* genetic background. This may be explained by the observation that RGL3's mRNA levels are positively regulated by ABA and low GA but to a lesser extent than those of RGL2. This could ensure that RGL2's repressive activity dominates relative to that of RGL3 under most germination conditions.

The mature Arabidopsis seed consists of a protective outer layer of dead tissue, the testa, underneath which the endosperm, a single layer of cells, surrounds the embryo.<sup>1</sup> When the seed germinates, the earliest visible event is that of testa rupture, soon followed by endosperm rupture (Fig. 1A). Testa rupture is visible as one or several slits forming at the surface of the seed. Endosperm rupture, usually chosen as the criteria for seed germination, is scored after the embryonic radicle tip emerges out of the testa. Such rupture events likely involve sugar bond modifying enzymes such as glucanases and mannanases<sup>2,3</sup> but in Arabidopsis they remain to be identified.

Endosperm rupture is antagonistically controlled by gibberellins (GA) and abscisic acid (ABA). GA promotes endosperm rupture whereas ABA represses it. We recently reported that low GA levels prevent endosperm rupture because they lead to the overaccumulation of the DELLA factor RGL2 that promotes an increase in endogenous ABA levels. In turn, ABA sustains high levels of ABI5 protein, a basic domain/leucine zipper transcription factor. ABI5 then acts to repress endosperm rupture. We therefore proposed that ABI5 is the final and common repressor of seed germination (i.e., when defined as endosperm rupture) in responses to changes

in both GA and ABA levels. A critical observation of our report is that RGL2's key role during seed germination arises from the strong positive regulation of its mRNA levels by ABA, either provided exogenously or endogenously as a result of the inhibition of GA synthesis.<sup>4</sup> This allows RGL2 protein levels to dominate relative to that of other DELLA factors such as RGA and GAI.<sup>4</sup>

As for endosperm, testa rupture is also antagonistically controlled by GA and ABA. We reported that RGL2 represses testa rupture in response to changes in GA and ABA levels. Indeed, testa rupture in *rgl2* mutants is insensitive to low GA or high ABA conditions.<sup>4</sup> Here we show that RGL3, a DELLA factor with previously unknown function, participates to repress testa rupture. However, this activity can only be observed in the absence of RGL2 (as in an *rgl2* mutant background). We postulate that this might also be due to the lower RGL3 protein abundance relative to that of RGL2. This is indeed suggested by the observation that RGL3 mRNA levels respond to ABA but to a much lesser extent than RGL2.

## RGL3 Expression is Stimulated by Both ABA and Inhibition of GA Synthesis

To define the role of RGL3 during seed germination we performed a time course of its mRNA accumulation upon seed imbibition in WT (Col) seeds in absence or presence of PAC, an inhibitor of GA synthesis, and ABA. We found that, similarly to RGL2, RGL3 mRNA levels transiently raised and decayed upon normal seed imbibition (Fig. 1B). Similarly to RGL2 as well, RGL3 mRNA levels were induced and maintained by ABA and PAC treatment. However, this stimulation was significantly more modest than that observed for RGL2<sup>4</sup> (compare with Fig. 1A<sup>4</sup>).

## RGL3 is Necessary to Repress Testa Rupture in Response to Both High ABA levels and Inhibition of GA Synthesis

The similar patterns of expression of RGL2 and RGL3 led us to investigate whether RGL3 function overlaps with that of RGL2 to repress testa rupture. We therefore compared the velocity of testa rupture events in WT and *rgl3* seeds. For comparison, we included *rgl2* mutant seeds, since they rupture testa faster under low GA or high ABA conditions.<sup>4</sup> We also included *rgl2/rgl3* double mutant seeds to reveal potential redundant effects between RGL2 and RGL3.

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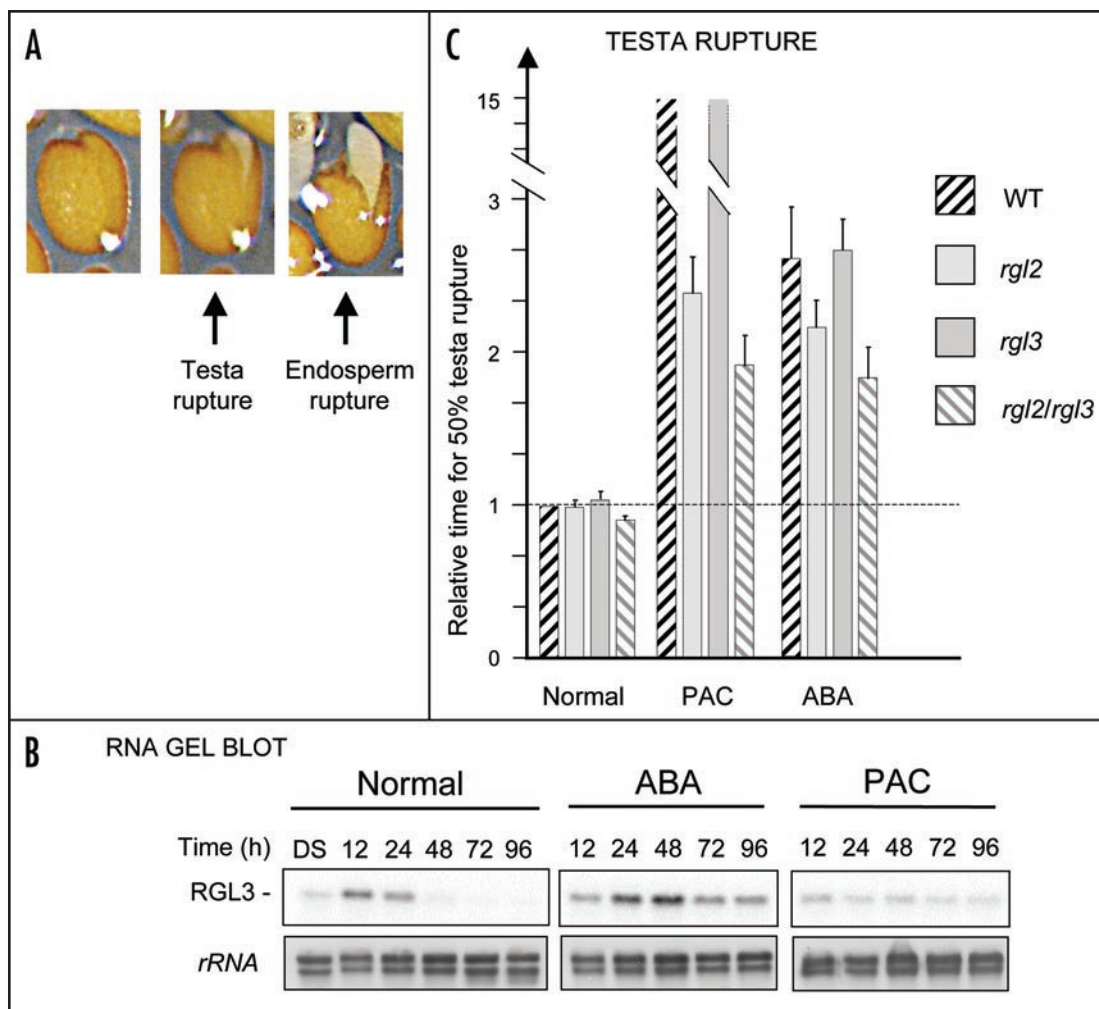


Figure 1. RGL3 expression responds to low GA and ABA conditions. RGL3 is necessary to repress testa rupture. (A) WT seed (Col) at different times upon imbibition showing testa and endosperm rupture events. (B) Northern blot analysis of a time course of *RGL3* mRNA levels upon WT (Col) seed imbibition in absence (Normal) or presence of 5  $\mu$ M paclobutrazol (PAC) or 5  $\mu$ M ABA (ABA). Hybridization signals can be directly compared between different conditions. 2  $\mu$ g of total RNA per lane. rRNA, ribosomal RNA; DS, dry seeds. (C) Velocity of testa rupture events in WT, *rgl2-13*, *rgl3-3* and *rgl2-13/rgl3-3* seeds was measured as the time needed for completion of testa rupture in half (50%) of the seed population. The time measured for the WT seed populations under normal germination conditions was used as a reference (=1) for relative comparison with all other time measurements obtained with other seed genetic backgrounds or germination conditions. PAC (5  $\mu$ M), ABA (25  $\mu$ M).

Under normal conditions, *rgl2* and *rgl3* seeds ruptured testa similarly to WT seeds with 50% of the population having a ruptured testa 28.5 hours after imbibition (Fig. 1C; this time is taken as a reference (=1) relative to all the other measurements shown in C). However, *rgl2/rgl3* seed populations reached this stage 3 hours earlier ( $t < 0.05$ ). This suggested that RGL2 and RGL3 redundantly repress seed germination under normal conditions.

In presence of PAC, testa rupture in WT and *rgl3* seeds was completely blocked (Fig. 1C). In contrast, testa rupture took place in PAC-treated *rgl2* seeds although the process was still delayed relative to normal conditions, as previously reported<sup>4</sup> (Fig. 1C). However, testa rupture occurred faster in PAC-treated *rgl2/rgl3* seeds than in *rgl2* seeds (Fig. 1C). These data indicate that RGL3 participates to repress testa rupture when GA levels are low but this is visible only when RGL2 is absent (as in an *rgl2* mutant background).

Testa rupture was similarly delayed in ABA-treated WT and *rgl3* seeds (Fig. 1C). Under these conditions, *rgl2* seeds ruptured their testa faster as previously reported<sup>4</sup> (Fig. 1C). However, testa rupture

took place even faster in *rgl2/rgl3* seeds (Fig. 1C). Taken together, these data are consistent with the notion that RGL3 is also necessary to repress testa rupture in response to ABA but again this is visible only when RGL2 is absent.

## Conclusions

The salient finding of this addendum to our recent report<sup>4</sup> is that RGL3 does play a role to repress testa rupture in response to changes in GA and ABA levels. However, RGL3's repressive activity appears to be dwarfed by that of RGL2 as it can only be revealed in an *rgl2* mutant background. We postulate that this is ultimately due to the modest responsiveness of *RGL3*'s mRNA levels to ABA. Indeed, it is significantly weaker than the one shown by *RGL2*.<sup>4</sup> Thus, exogenous ABA or PAC treatment, which increases endogenous ABA levels, maintain *RGL3* mRNA over time but they fail to significantly surpass the peak levels observed 12 hours after imbibition under normal conditions. As a result, RGL3 protein levels relative to RGL2 may decrease, like those of GAI and RGA,<sup>4</sup> so that RGL2's

repressive activity dominates relative to that of all the other DELLA factors. The observation that RGL2 and RGL3 redundantly repress testa rupture under normal conditions is significant in this respect. Indeed, normal conditions are those under which *RGL3* mRNA levels reach their highest levels relative to those of *RGL2*. This occurs between 12 h and 48 h after imbibition, which is precisely the time when testa rupture is taking place. Thus it is tempting to speculate that RGL3 protein levels are sufficiently high under normal conditions to redundantly delay testa rupture together with RGL2.

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