

ARTICLE ADDENDUM



## Regulatory-Associated Protein of TOR 1B (RAPTOR1B) regulates hormonal switches during seed germination in *Arabidopsis thaliana*

Mohamed A. Salem<sup>a,b</sup> and Patrick Giallisco<sup>b\*</sup>

<sup>a</sup>Department of Pharmacognosy, Faculty of Pharmacy, Menoufia University, Shihin Elkom, Egypt; <sup>b</sup>Max Planck Institute of Molecular Plant Physiology, Germany

### ABSTRACT

Target of Rapamycin (TOR) regulates multiple growth- and metabolic-related processes in *Arabidopsis thaliana* as in all other eukaryotes. While several of these processes have been investigated in diverse *Arabidopsis* growth stages, little is known about hormonal and metabolic regulation of TOR during seed germination. This is mainly due to the fact that *Arabidopsis* knockout lines of TOR are embryo lethal. Here, we utilized the knockout lines of TOR-interacting protein, *REGULATORY-ASSOCIATED PROTEIN OF TOR 1B (RAPTOR1B)*, to perform comprehensive hormone profiling during seed germination. We previously reported that RAPTOR1B positively regulates seed germination by maintaining the nutritional and hormonal balance. In the current analysis, dry and imbibed seeds as well as germinated seeds were subjected to detailed hormone analysis. Accordingly, the abscisic acid content of dry and imbibed *raptor1b* seeds was higher than that of WT, while the amounts of gibberellins were comparable after stratification. Further analysis showed that *raptor1b* seeds maintained higher levels of indole-3-acetic acid and jasmonates, namely jasmonic acid (JA) and 12-oxo-phytodienoic acid, even after stratification. The combination of this hormonal perturbation seems to be the driving factor for the observed delayed germination phenotypes in *raptor1b* seeds.

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### Overview

Plant germination, growth, and development are the results of interaction between environmental cues and diverse endogenous signals.<sup>1</sup> A key regulator that integrates cellular growth in response to external stimuli is TOR, which is well conserved among all eukaryotes and thoroughly studied in animals and yeast.<sup>2,3</sup> TOR assembles into two protein complexes commonly termed TOR complex 1 and 2 (TORC1 and TORC2).<sup>4</sup> Plants seem to only contain the core members of TORC1, namely RAPTOR and lethal with sec thirteen protein 8 (LST8).<sup>5,6</sup> One TOR gene, two RAPTOR genes (*RAPTORIA* and *RAPTOR1B*), and two LST8 genes (*LST8-1* and *LST8-2*) have been annotated from the *Arabidopsis* genome.<sup>5–8</sup> Both RAPTOR genes are actively transcribed, while only *LST8-1* seems to be actively expressed.<sup>5,6,8</sup> Additionally, *raptor1b* (At3g08850) and *lst8-1* (At3g18140) mutants show significant and almost similar growth, developmental and metabolic phenotypes.<sup>5,6,9</sup> Moreover, disruption of the *RAPTORIA* has no visible phenotypes,<sup>8</sup> while the lack of detectable expression of *LST8-2* indicated that it is a nonfunctional gene.<sup>5</sup>

As mentioned earlier, TOR acts as a central hub that is involved in multiple processes associated with plant growth and proliferation.<sup>10</sup> In contrast to the embryo lethality of *tor*-mutant lines, the knockout lines of the TOR-interacting partners,


*RAPTOR1B* (At3g08850) and *LST8-1* (At3g18140), are viable.<sup>5,6</sup> *Arabidopsis* eFP Browser data suggested the preferential expression of TOR complex genes, namely *TOR*, *RAPTOR1B*, and *LST8-1* in dry seeds, indicating potential roles of the TOR complex in developing and maturing seeds.<sup>11</sup> In recent papers, the consequences of *RAPTOR1B* mutation on seed and vegetative tissue physiology and metabolism were investigated.<sup>9,12</sup> RAPTOR1B was reported to control some physiological and molecular aspects of *Arabidopsis* seeds and vegetative tissue to maintain the proper morphology, anatomy, and metabolic content.<sup>9,12</sup> Previous data revealed that TOR is a key player in regulating phytohormone signaling pathways in *Arabidopsis* growth in leaf and root tissues.<sup>13–17</sup> However, little is known about RAPTOR1B function during the process of seed germination. Here, we report that RAPTOR1B modulates hormonal homeostasis during seed germination. We analyzed the hormone content of WT *Arabidopsis* seeds and *raptor1b* during the different germination stages. For this purpose, we harvested samples from six stages, namely, dry seeds, seeds after 72 h of imbibition at 4°C and 1, 2, 3, and 4 days after imbibition. The later samples represent seeds that were already germinated. Results of this analysis revealed that the high abscisic acid (ABA), indole acetic acid (IAA), and jasmonic acid (JA) constitute the central node for the observed delayed germination of *raptor1b* seeds.

**CONTACT** Mohamed A. Salem ✉ [Salem@mpimp-golm.mpg.de](mailto:Salem@mpimp-golm.mpg.de) Department of Pharmacognosy, Faculty of Pharmacy, Menoufia University, Egypt

\*Current address: Max Planck Institute for Biology of Ageing, Joseph Stelzmann Str. 9b, 50931 Cologne, Germany

### Addendum to:

Salem MA, Li Y, Wiszniewski A, Giallisco P. Regulatory-associated protein of TOR (RAPTOR) alters the hormonal and metabolic composition of *Arabidopsis* seeds, controlling seed morphology, viability and germination potential. *Plant J* 2017; 92:525–45.

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## Mutation of RAPTOR1B delays seed germination

Based on the recently published phenotypic observations of *raptor1b* seeds,<sup>12</sup> we further dissected the role of RAPTOR1B during Arabidopsis seed germination. For this purpose, we employed the same two independent *raptor1b* lines (*rb10* and *rb22*) that were used in our previous study,<sup>12</sup> and compared them to their respective WT. To investigate the effect of RAPTOR1B on seed dormancy, the germination rate of freshly harvested seeds of WT and *raptor1b* was scored. It became obvious that the germination rate of *raptor1b* seeds was significantly lower than WT without stratification treatment (Figure 1(a)).

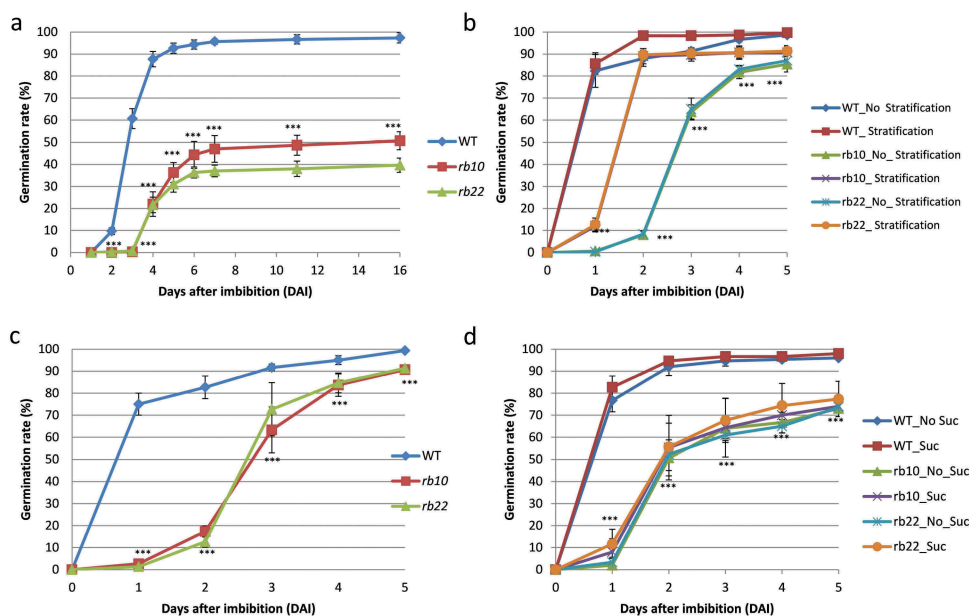
Previous studies have shown that dormancy can be released by extended dry seed storage (after-ripening) or stratification treatment at low temperature.<sup>18,19</sup> Therefore, we also investigated the effect of three month-dry storage with or without stratification. Without stratification, only 1% of the *raptor1b* seeds germinated on the first day after imbibition (DAI) compared to 82% germination efficiency of WT seeds. Interestingly, *raptor1b* seeds reached the 82% germination efficiency only after 4 DAI (Figure 1(b)). When seeds, that were fully after-ripened, were stratified for 3 days, the delayed germination of *raptor1b* seeds was reduced from 3 to 1 DAI. Accordingly, 10% of the *raptor1b* seeds germinated 1 DAI compared to 86% germination efficiency of WT seeds; however, after-ripened and stratified *raptor1b* seeds reached the 89% germination efficiency 2 DAI (Figure 1(b)). This indicates that the differences between WT and *raptor1b* seeds decreased significantly with stratification.

It was also reported that nitric oxide (NO), triggered by CN<sub>2</sub>, nitrate, or nitrite, promotes a loss of dormancy.<sup>20,21</sup> Nitrate can inhibit ABA synthesis and enhance ABA catabolism; therefore, we also investigated the effect of external

application of 7 mM KNO<sub>3</sub>.<sup>21</sup> The results of the time-course germination scoring showed that nitrate treatment was not able to improve the germination rate of *raptor1b* seeds (Figure 1(c)). Additionally, we reported previously that *raptor1b* seeds showed significantly lower sucrose level in their dry seeds,<sup>12</sup> which prompted us to test the influence of external application of sucrose on the germination rate. Surprisingly, contrary to the increased greening efficiency of etiolated *raptor1b* seedlings after sucrose feeding,<sup>22</sup> no differences were detected in the germination efficiencies of *raptor1b* seeds with or without sucrose supplementation (Figure 1(d)). Accordingly, it can be concluded that *raptor1b* seeds germinated slower and at a lower frequency compared to WT seeds even in presence of sucrose, indicating that this phenotype is, contrary to the sugar-dependent greening after extended etiolation,<sup>22</sup> a sugar-independent phenotype (Figure 1(d)). Furthermore, *raptor1b* seeds showed, beyond their delayed germination, less vitality, consistent with our previous observation.<sup>12</sup>

## RAPTOR1B controls hormonal balance during seed germination

We previously showed that a mutation in RAPTOR1B is associated with perturbation of hormonal levels especially increased levels of ABA, IAA, and jasmonates.<sup>12</sup> In the previous publication,<sup>12</sup> we were not able to quantify gibberellins (GAs) level in dry seeds due to their low concentration. The endogenous level of the bioactive GA (GA<sub>4</sub>) is often not detectable in dry Arabidopsis seeds even if additional sample purification steps are employed.<sup>23</sup> However, we have shown that external GA application can partially rescue the delayed germination phenotypes of *raptor1b* seeds.<sup>12</sup> It is known that



**Figure 1.** Delayed germination of *raptor1b* seeds.

A. *raptor1b* seeds have altered dormancy. Germination rate was scored from freshly harvested, non-stratified seeds. B. Germination of fully after-ripened WT and *raptor1b* seeds with or without stratification treatment. C. External application of nitrate did not improve the germination of *raptor1b* seeds. D. *raptor1b* seeds showed delayed germination with or without external supplementation of sucrose. Error bars indicate mean  $\pm$  SD for 5 biological replicates. Black asterisks indicate significantly different from WT ( $P < .001$ , Student's *t*-test). Stratification refers to seeds that have been kept for 3 days at 4°C. Values of germination percentages are from at least 5 biological replicates of 100 seeds each (100 seeds/plate/5 replicates).

stratification of *Arabidopsis* seeds is associated with increased GA level.<sup>24</sup> Therefore, we repeated our analysis, starting from 200 mg of stratified seed material and included three solid phase extraction (SPE) steps,<sup>23</sup> to purify and enrich for GA. Because of the phenotypic similarity between the two *raptor1b* lines,<sup>12</sup> only one line (*rb10*) was chosen for the extraction, purification, and analysis of GA. Intriguingly, the endogenous level of GA did not show significant changes between *raptor1b* and WT seeds (Figure 2).

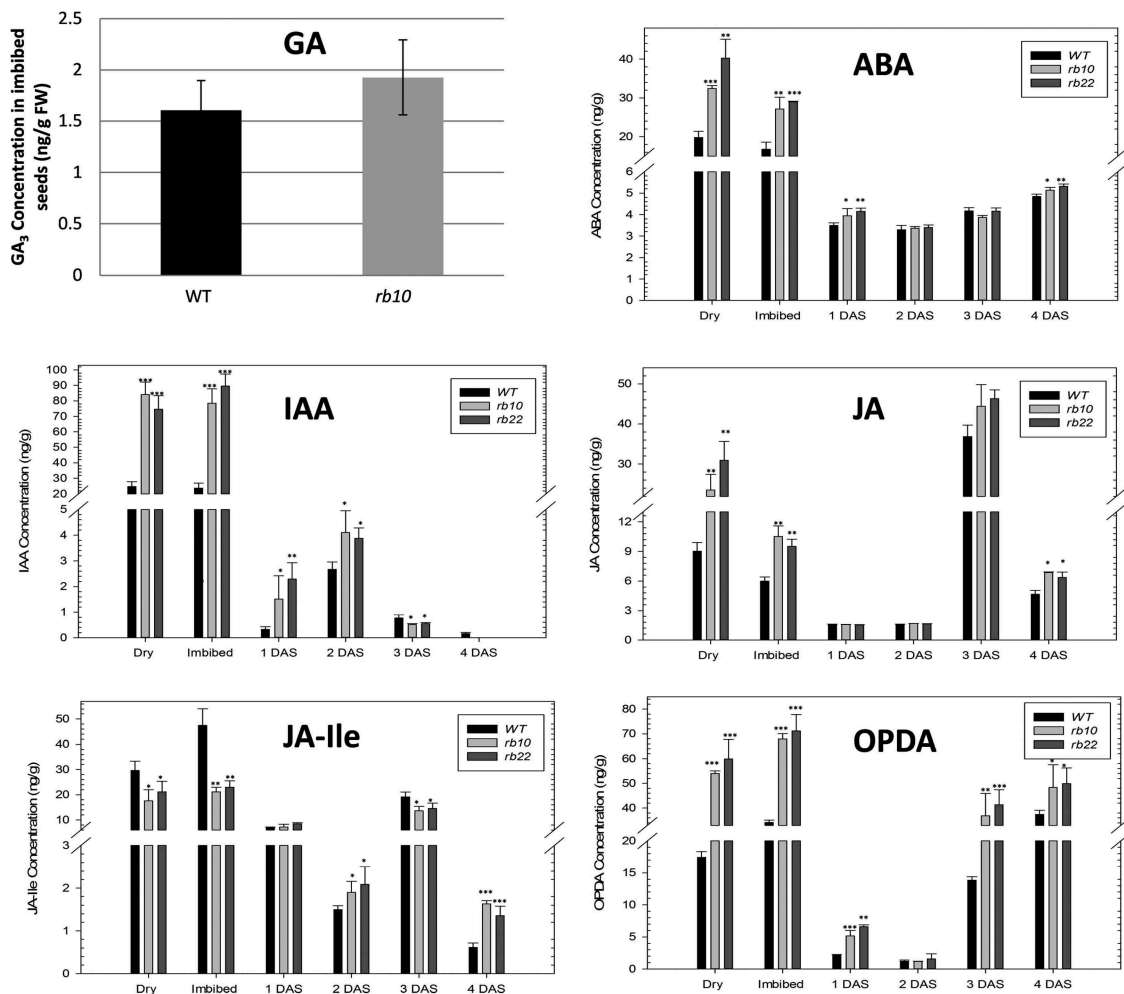
To obtain an overview of other hormonal changes occurring during the transition from dry seeds to imbibed seeds as well as the dynamics during germination, we comprehensively analyzed the hormone levels of WT and *raptor1b* dry, imbibed, and germinated seeds. ABA levels decreased partially with seed imbibition in WT seeds, this was also true for *raptor1b* seeds (Figure 2). However, *raptor1b* showed massively increased ABA level in both dry and imbibed seeds. This level stayed also significantly higher after one day of seed germination. Interestingly, after radical emergence and cotyledon growth, both WT and *rb* seeds reached similar level. IAA levels did not change with seed imbibition in WT and *raptor1b* seeds. However, *raptor1b* showed significantly higher IAA level in both dry and imbibed seeds. This level significantly decreased after one day of seed germination. *raptor1b*

seeds showed higher IAA level at 1 and 2 DAS. JA and its precursor OPDA showed also significantly higher level in both dry and imbibed seeds (Figure 2).

## Conclusion and perspectives

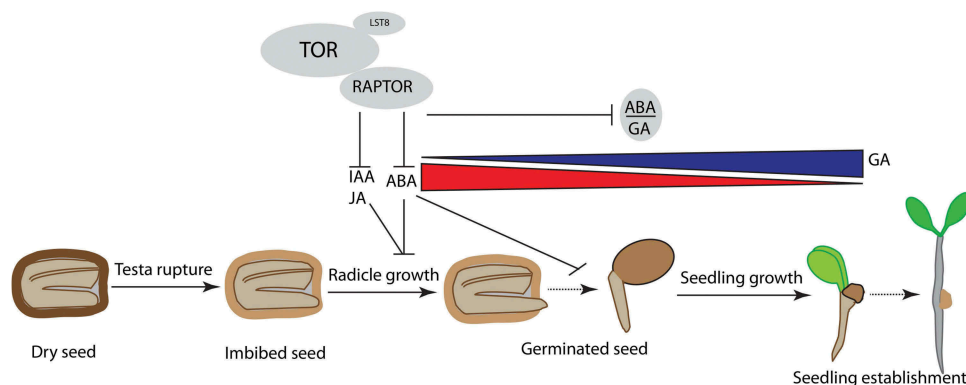
The hormones ABA and GA are known to control seed germination through antagonistic action, where ABA promotes seed dormancy and inhibits germination, while GA pursues the opposite. Previous data showed that the ABA/GA ratio, rather than the absolute amount of ABA or GA, plays a crucial role in releasing dormancy and promoting germination.<sup>25</sup>

In the present study, the *raptor1b* seeds showed a delayed germination phenotype of fresh seeds but also with seeds that were subjected to a 3-month dry storage. This delayed germination was partially improved by stratification. Furthermore, stratification reduces ABA content in the same way in both WT and *raptor1b* seeds. However, *raptor1b* imbibed seeds have significantly higher ABA level (~1.6–1.7 fold increase) than WT. Contrary to the high ABA level in seeds, recent data showed that *lst8* and *raptor1b* mutants showed a significantly decreased ABA level in vegetative tissue, indicating the tissue specificity of RAPTOR1B hormonal regulation.<sup>9,26</sup> Beyond the differential ABA concentrations, the GA level in WT and



**Figure 2.** Quantification of hormones in *raptor1b* and WT seeds.

Error bars indicate mean  $\pm$  SD for 5 biological replicates. Black asterisks indicate significantly different from WT (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , Student's  $t$ -test).



**Figure 3.** RAPTOR1B positively regulates seed germination in *Arabidopsis thaliana*.

*raptor1b* seeds was overall comparable, indicating that the increased ABA/GA ratio is most likely a result of higher ABA levels in the mutant seeds. Accordingly, this hormonal measurement seems to reveal that the increased ABA level in dry *raptor1b* seeds contributed to the delayed germination phenotype.

Other hormones have been reported to crosstalk with ABA and/or GA.<sup>27</sup> Auxins, for example, induced ABA-dependent seed dormancy,<sup>28</sup> while JA and its metabolites are also known to have a negative impact on seed germination.<sup>18,29</sup> Accordingly, the emerging finding concerning the hormonal crosstalk of other germination-delaying hormones, namely, IAA and JA support the speculation that the hormonal imbalance associated to these three hormonal classes, was the most obvious molecular phenotype of *raptor* seeds.

During seed development, diverse compounds accumulated including proteins, lipids, and carbohydrates.<sup>30</sup> Reserve mobilization and metabolism activation start upon imbibition.<sup>31</sup> This metabolic activation is influenced also by hormonal levels. For instance, it was shown that lipid mobilization is repressed by ABA INSENSITIVE4 (ABI4) during germination.<sup>32</sup> Previous data showed that seed imbibition was associated with reduction in most seed metabolites that implies the support of a metabolic switch toward seed germination.<sup>33</sup> Our recent report indicated that *raptor1b* showed higher accumulation of amino acids and sugars in their dry seeds.<sup>12</sup> This result indicates that, even though *raptor1b* seeds contained sufficient amount of reserve nutrients, they were unable to efficiently utilize these to support their efficient germination. This indicates that the availability of nutrients *per se* is not the limiting factor for the delayed germination. Apparently, hormonal imbalance controls or correlates with the inability of *raptor1b* seeds to efficiently metabolize their reserve nutrients.

Taken together, the present study confirms that RAPTOR1B and hence TOR<sup>9</sup> positively regulate seed germination by maintaining the hormonal balance, specifically the ABA/GA ratio and the content of other germination-delaying hormones such as IAA and JA.<sup>28,34,35</sup> Further, this study suggests and opens the possibility that RAPTOR1B plays crucial and complex roles in the cross-talk between hormones during germination; however, more detailed molecular dissection is necessary to obtain a deeper understanding of this cross-talk and the driving forces behind it (Figure 3). Here especially metabolic and hormonal profiling associated to detailed proteomic and transcriptomic studies during the

stages of developing seeds (seed maturation), where most of the reserve accumulation is happening, in addition to the transition from seed maturation to seed desiccation might provide more detailed insight.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## ORCID

Patrick Gialvalisco  <http://orcid.org/0000-0002-4636-1827>

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