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Letters

ABA INSENSITIVE4 promotes rather than represses PHYA-dependent seed germination in *Arabidopsis thaliana*

Light quality plays vital roles in the life cycle of plants. For example, in seeds of many species, light quality determines the levels of the gibberelic acid (GA) and abscisic acid (ABA) phytohormones, which promote and repress seed germination respectively (Seo *et al.*, 2006). In *Arabidopsis thaliana*, the photoreceptors phytochrome A (PHYA) and phytochrome B (PHYB) distinguish between full light (rich in red wavelength; R) and shade light (rich in far-red wavelength; FR) to regulate seed germination (Lymperopoulos *et al.*, 2018). PHYB is reversibly activated and deactivated by R and FR light, respectively. Unlike the effect upon PHYB, both R and FR light irreversibly activate PHYA and once active, PHYA is more resistant to proteasome-mediated degradation (Shinomura *et al.*, 1994, 1996 Debrieux & Fankhauser, 2010). Both PHYA and PHYB promote germination by targeting the transcription factor PHYTOCHROME INTERACTING FACTOR 1 (PIF1) for protein degradation (Shen *et al.*, 2005; Oh *et al.*, 2006). When PHYA and PHYB are inactive PIF1 accumulates and regulates expression of genes leading to low GA/ABA ratios, which in turn repress germination (Oh *et al.*, 2004, 2007; Kim *et al.*, 2016). Conversely, upon PHYA and PHYB activation, and the subsequent PIF1 degradation, GA/ABA ratios increase to promote germination.

In addition to their different light-quality dependent activation, PHYA and PHYB have distinct patterns of accumulation: while PHYB accumulates from the beginning of seed imbibition; PHYA only accumulates after longer imbibition periods (Lee *et al.*, 2012). The fact that there are differences in response to light and accumulation patterns between PHYA and PHYB has been used to dissect their functions: short pulses of R and FR light at early stages of seed imbibition (before PHYA accumulates) are sufficient to reversibly activate and deactivate PHYB. Hence, two consecutive FR and R light pulses (FR/R; Fig. 1a) activate PHYB. By contrast, only one FR pulse (FR; Fig. 1a) deactivates PHYB. Exposure to FR light at later stages of imbibition activates PHYA, but still deactivates PHYB. Thus, an initial short FR light pulse followed later by a 60 min long FR light exposure (FR/FR; Fig. 1a), or a continuous 48 h FR light treatment (FR48; Fig. 1a), results in activation of PHYA and deactivation of PHYB.

ABA acts through the signalling factors ABA INSENSITIVE3 (ABI3), ABI4 and ABI5, which are B3-, AP2- and bZIP-type transcription factors, respectively (Finkelstein *et al.*, 1998; Finkelstein & Lynch, 2000; Clerkx *et al.*, 2003). These three factors were originally identified as mutations that resulted in seeds that were insensitive to ABA treatments (Koornneef *et al.*, 1984; Finkelstein, 1994). The corresponding genes were later found to also be involved in ABA signalling in other biological processes distinct from seed germination (Rohde *et al.*, 2000; Signora *et al.*, 2001; Rushton *et al.*, 2012). The roles of ABI3 and ABI5 in light-dependent seed germination have been described previously: *ABI3* gene expression is induced under PHYB deactivating light conditions and, in turn, ABI3 controls expression of ABA-response related genes including *ABI5* (Piskurewicz *et al.*, 2009). ABI5 plays more of a secondary role with a relatively modest effect under light conditions leading to PHYA activation and PHYB deactivation (Lee *et al.*, 2012). This is probably due to the fact that the jasmonic acid precursor oxylipin *cis*-12-oxo-phytodienoic acid (OPDA) also plays a critical role in light-quality dependent repression of seed germination in an ABI5 independent manner (Barros-Galvão *et al.*, 2019).

Regarding ABI4, it has been shown to control lipid metabolism in seeds, sugar-directed growth arrest, lateral root development, and plastid-to-nucleus retrograde signalling (Wind *et al.*, 2013). Intriguingly, previously published transcriptomic analysis revealed that, opposite to expectation, *ABI4* gene expression is repressed by FR light conditions, which are known to increase ABA levels (Oh *et al.*, 2009; Vaistij *et al.*, 2018). These observations prompted us to investigate the role of ABI4 on the light-quality dependent germination pathway. We first compared *ABI3*, *ABI4* and *ABI5* gene expression upon FR/FR light treatment in *phya-211* mutant seeds, which do not germinate under these conditions (Lee *et al.*, 2012). Twelve hours after the end of the second FR light treatment (61 h after imbibition, hai; Fig. 1a) *ABI3* and *ABI5* expression was, as expected, increased in mutant seeds compared to wild-type control seeds (Fig. 1b). By contrast, *ABI4* expression was repressed in *phya-211* seeds (Fig. 1b). This shows that PHYA, an inducer of germination, promotes *ABI4* expression. We then assessed germination of lack-of-function *abi4-1* mutant seeds (120 hai; Fig. 1a). We also analysed seeds of an *abi4-1* complemented line (*abi4-1*comp). As expected, under FR/R conditions, seeds of all genetic backgrounds analysed germinated at similar high rates (Fig. 1c,d). Under FR light conditions, germination of all seeds was severely repressed (Fig. 1c,d). By contrast, under FR/FR and FR48 light conditions, while wild-type and *abi4-1*comp seeds germinated at relatively high and similar levels, *abi4-1* mutant seeds germinated at lower rates (Fig. 1c,d). These observations demonstrate that, in contrast to what has been reported for ABI5 (Lee *et al.*, 2012), ABI4 promotes PHYA-dependent germination.

Previous studies showed that ABI4 and ABI5 are not only involved in ABA signalling, but they also positively feedback to regulate expression of genes leading to reduced GA/ABA ratios

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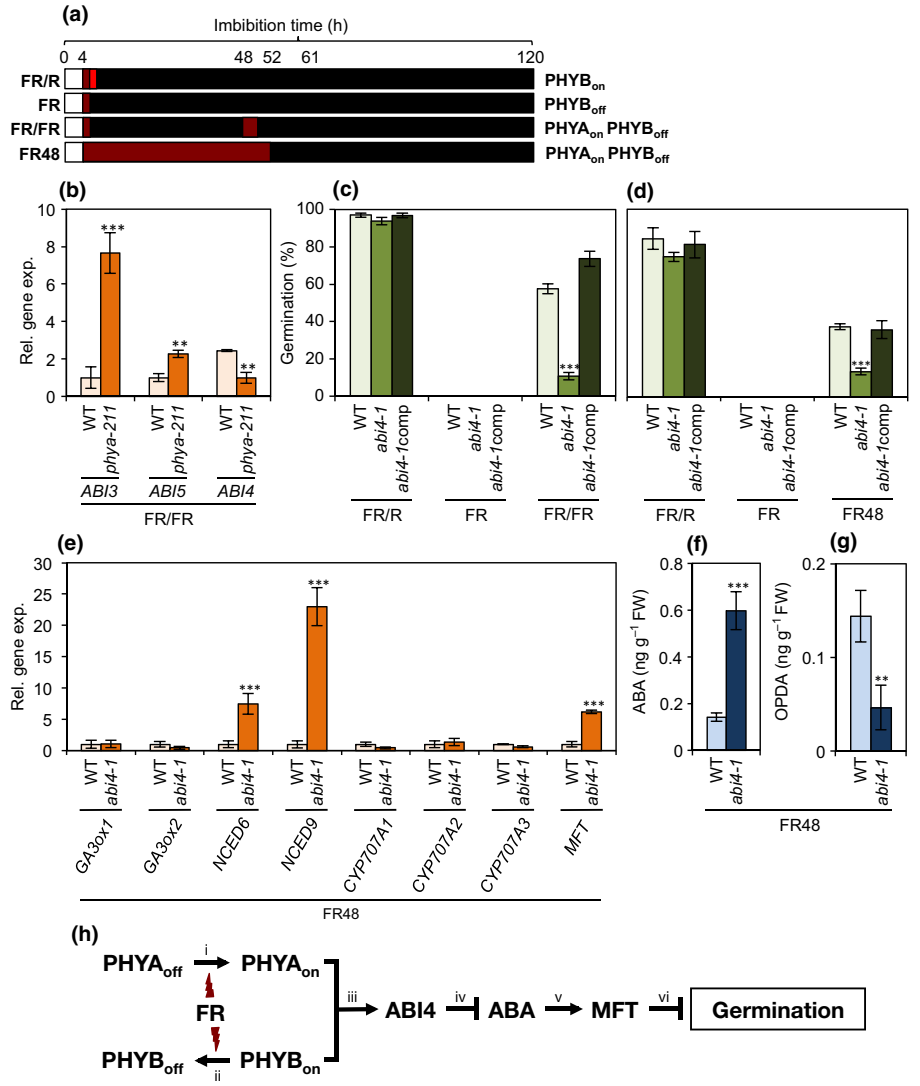

Fig. 1 ABSCISIC ACID INSENSITIVE 4 (ABI4) promotes phytochrome A (PHYA)-dependent germination and represses both abscisic acid (ABA) accumulation and MOTHER-OF-FT-AND-TFL1 (*MFT*) gene expression in *Arabidopsis thaliana*. (a) Schematic of the experimental set up. Seeds were imbibed on water-agar plates for 4 h under low light and then treated with: (1) two successive 5 min far-red (FR) and red (R) light pulses to activate PHYB (PHYB$_{on}$) during the period when PHYA does not accumulate; (2) only one 5 min FR light pulse to deactivate PHYB (PHYB$_{off}$) during the period when PHYA does not accumulate; (3) an initial 5 min FR light pulse followed 44 h later (48 h after imbibition, hai) by a second 60 min long FR light exposure to activate PHYA and deactivate PHYB (PHYA$_{on}$ PHYB$_{off}$); or (4) a continuous FR exposure for 48 h (FR48) to activate PHYA and deactivate PHYB (PHYA$_{on}$ PHYB$_{off}$). Seeds were kept in the dark between and after light treatments. (b) Relative expression of *ABI3*, *ABI5* and *ABI4* in FR/FR treated wild-type (WT) and *phya-211* seeds 12 h after the end of the second FR treatment (61 hai). (c, d) Germination (120 hai) of FR/R, FR and FR/FR (c) and FR/R, FR and FR48 (d) treated WT, *abi4-1* and *abi4-1*comp seeds. (e) Relative expression of *GA3ox1*, *GA3ox2*, *NCED6*, *NCED9*, *CYP707A1*, *CYP707A2* and *CYP707A3* in FR48 treated WT and *abi4-1* seeds (52 hai). ABA (f) and 12-oxo-phytodienoic acid (OPDA) (g) levels in FR48 treated WT and *abi4*-1 seeds (52 hai). Data are means ± SD of three (for gene expression) and four (for germination and phytohormones levels) biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's *t*-test (**, $P < 0.01$; ***, $P < 0.001$). (h) Model of the role of PHYA and ABI4 in the promotion of seed germination: FR light (FR/FR and FR48 treatments in our experimental set up) activates PHYA (PHYA$_{on}$) (i) and deactivates PHYB (PHYB$_{off}$) (ii). PHYA$_{on}$ promotes *ABI4* gene expression (iii). ABI4 represses accumulation of ABA (iv) by inhibiting expression of the ABA-biosynthesis *NCDE6* and *NCDE9* genes (not depicted in the model). ABA acts, at least partially, through MFT (v) to repress seed germination (vi).

(Lee *et al.*, 2012; Shu *et al.* 2013, 2016). This led us to assess whether expression of key genes involved in GA-biosynthesis (*GA3ox1* and *GA3ox3*), ABA-biosynthesis (*NCED6* and *NCDE9*), and ABA-breakdown (*CYP701A1*, *CYP701A2* and *CYP701A3*) are regulated by ABI4 under FR48 light conditions (52 hai; Fig. 1a). We found that, while expression of GA-biosynthesis and ABA-breakdown related genes were unchanged, ABA-biosynthesis genes were up regulated in *abi4-1* seeds (Fig. 1e). This prompted us to measure ABA levels in FR48 light treated seeds (52 hai; Fig. 1a). We found that, in accordance with the increased *NCED6* and *NCED9* expression, ABA was present at higher levels in *abi4-1* compared to wild-type seeds (Fig. 1f). These results show that, in contrast to its role in promoting ABA accumulation in post-germination developmental stages (Shu *et al.*, 2016), ABI4 represses ABA accumulation during PHYA-dependent promotion of seed germination.

We reported previously that OPDA is a key repressor of germination (Dave *et al.*, 2011, 2016). More recently we also showed that, under FR light conditions, and to a lesser extent under FR48 light conditions, OPDA acts in parallel to the action of ABA (Barros-Galvão *et al.*, 2019) in repressing seed germination. Hence, we also measured OPDA and found that levels were decreased in *abi4-1* seeds (Fig. 1g). In our previous study, we showed that the abnormally high germination of ABA and OPDA deficient mutant seeds is repressed by either ABA or OPDA treatments (Barros-Galvão *et al.*, 2019). Presumably, in the present study the high ABA levels that lead to germination inhibition compensate for the low OPDA levels in *abi4-1* seeds.

In two previous studies from our laboratory, we demonstrated that MOTHER-OF-FT-AND-TFL1 (MFT) is a repressor of seed germination, that it is a key factor of the ABA-signalling pathway, and that *MFT* gene expression is promoted by shade light (Vaistij *et al.*, 2013, 2018). This prompted us to assess whether *MFT* expression is regulated by ABI4. We found that, under FR48 light conditions (52 hai; Fig. 1a), *MFT* expression was increased in *abi4-1* seeds (Fig. 1e). This observation shows that, as for *NCDE6* and *NCDE9*, ABI4 inhibits *MFT* expression. Whether this repression is due to direct or indirect ABI4–*MFT*/*NCDEs* interactions remains to be determined.

In conclusion, previous studies have established that ABI3, ABI4 and ABI5 are ABA-signalling effectors repressing germination (Koornneef *et al.*, 1984; Finkelstein, 1994). It has also been demonstrated that ABI3 and ABI5 act under shade light conditions to inhibit germination (Piskurewicz *et al.*, 2009; Lee *et al.* 2012). In the current study, we reveal an unexpected role for ABI4 in promoting, rather than repressing, PHYA-induced germination (Fig. 1h): upon light activation, PHYA promotes *ABI4* gene expression (probably through the action of PIF1). In turn, ABI4 represses expression of *NCDE6* and *NCDE9*, which leads to a decrease in ABA levels in the seed. ABI4 also reduces *MFT* gene expression either directly or indirectly through its effect on ABA, which itself promotes *MFT* expression (Xi *et al.*, 2010). Interestingly, it has been reported that, as under PHYA activating light conditions (Fig. 1b), the pattern of expression of *ABI4* is opposite to that of *ABI3* and *ABI5* in both Arabidopsis seed dormancy cycling (Footitt *et al.*, 2011, 2014) and in *Aethionema arabicum* light-dependent seed germination (Mérai *et al.*, 2019). This suggests that the germination-promoting role of ABI4 is a more general phenomenon. The evolutionary processes resulting in ABI4 function switching from a repressor to a promoter of seed germination depending on the environmental conditions remain to be elucidated.

Methods

The mutant *abi4-1* and *phya-211* lines were described previously (Finkelstein, 1994; Reed *et al.*, 1994). The *abi4-1*comp line was obtained by transformation of the mutant line with a pGREEN derived binary vector (pGTI0242ΔGR) carrying the *ABI4* coding sequence under the control of the CaMV 35S promoter. All Arabidopsis lines used in this work are of the Columbia ecotype. Plant growth conditions, seed collection, germination assays, RNA extraction, primer sequences, quantitative polymerase chain reactions (qPCRs) conditions and phytohormones extractions were described previously (Dave *et al.*, 2016; Vaistij *et al.*, 2018; Barros-Galvão *et al.*, 2019). For gene expression analysis, transcript levels as determined by qPCR were normalized to *UBQ11* expression and expressed relative to the lower of the expression levels in each of the wild-type vs mutant comparisons.

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Author contributions

TB-G, FEV and IAG planned and designed the research. TB-G, AD, ADG, DH and FEV performed experiments and analysed data. TB-G, FEV and IAG wrote the manuscript.

ORCID

Thiago Barros-Galvão https://orcid.org/0000-0002-0112-7084
Anuja Dave https://orcid.org/0000-0003-1003-9403
Ian A. Graham https://orcid.org/0000-0003-4007-1770
Fabián E. Vaistij https://orcid.org/0000-0002-1469-0589

Thiago Barros-Galvão, Anuja Dave, Alison D. Gilday, David Harvey, Fabián E. Vaistij and Ian A. Graham*

Department of Biology, Centre for Novel Agricultural Products, University of York, York YO10 5DD, UK
(*Author for correspondence: tel +44 (0) 1904 328750; email ian.graham@york.ac.uk)

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