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Control of seed coat rupture by ABA-INSENSITIVE 5 in *Arabidopsis thaliana*

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Short Communication

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Abstract

In Arabidopsis, seed germination is a biphasic process involving rupture of the seed coat followed by emergence of the radicle through the micropylar endosperm. Embryo expansion results in seed coat rupture and removal of seed coat imposed dormancy with DELLA proteins blocking embryo expansion in the absence of gibberellins. Exogenous abscisic acid (ABA) treatment does not block seed coat rupture but does block radicle emergence. We used this limited effect of exogenous ABA to further investigate the mechanism by which it blocks the onset of germination marked by seed coat rupture. We show that physical nicking of the seed coat results in exogenous ABA treatment blocking both seed coat and endosperm rupture and this block requires the transcription factors ABI3 and ABI5, but not ABI4. Furthermore, we show that the repression of expression of several *EXPANSIN* genes (*EXPA1*, *EXPA2*, *EXPA3*, *EXPA9* and *EXPA20*) by exogenous ABA requires ABI5. We conclude that ABI5 plays an important role in the ABA-mediated repression of germination through prevention of seed coat rupture and propose that this involves EXPANSIN related control of cell wall loosening.

Introduction

Seed germination is a complex developmental process that marks the beginning of the life cycle of a higher plant. For over 40 years, seed biologists have understood that the balance between the inhibitory actions of abscisic acid (ABA) and the stimulating actions of gibberellins (GA) is the primary determinant of seed dormancy and germination (Luckwill, 1952; Karssen and Lącka, 1985; Footitt *et al*., 2011; Rajjou *et al*., 2012). In coat-imposed dormancy species, germination can be achieved via simple removal of or damage to the seed coat. Previous work has shown that the seed coat may restrict germination by controlling permeability to either water, oxygen or germination inhibitors that leach from the seed (Edwards, 1968; Wyatt, 1977; Corbineau and Côme, 1993). Arabidopsis seeds have a single endosperm cell layer between the embryo and the seed coat, and endosperm-imposed dormancy prevails over embryo- and seed coat-imposed dormancy with the phytohormones GA and ABA regulating germination in a tissue-specific manner (Penfield *et al*., 2004; Lee *et al*., 2010). ABA is biosynthesized mainly in the endosperm of dormant seeds and transported to the embryo in order to repress embryo expansion and therefore germination (Lee *et al*., 2010; Kang *et al*., 2015). However, Arabidopsis seeds still display some degree of coat-imposed dormancy, and alterations in seed coat development or pigmentation often result in reduced dormancy (Shirley *et al*., 1995; Debeaujon *et al*., 2000, 2003).

Upon imbibition, GA promotes Arabidopsis germination processes and ABA inhibits them (Müller *et al*., 2006; Piskurewicz *et al*., 2008). Arabidopsis seeds germinate in a two-step process that involves longitudinal splitting of the seed coat along the embryo axis followed by emergence of the radicle through the micropylar endosperm (Liu *et al*., 2005; Müller *et al*., 2006). Both these events require embryo expansion, with the latter generally considered as completion of seed germination. Under certain germination conditions or genetic backgrounds radicle emergence through both the endosperm and seed coat can occur prior to longitudinal splitting of the seed coat (Piskurewicz *et al*., 2008, 2009; Lee *et al*., 2012), demonstrating that control of the two phases of germination can be separated and therefore may be under separate control.

GA induces germination via destabilization of the DELLA proteins, which are negative regulators of GA-inducible genes (Lee *et al*., 2002; McGinnis *et al*., 2003; Tyler *et al*., 2004; Cao *et al*., 2005). The requirement for exogenous GA to promote seed coat rupture and germination in the *ga1-3* background, which is disrupted in GA biosynthesis, can be compensated for by loss of function mutations in the DELLA genes (e.g. *ga1-3 rgl2-1 gai-t6 rga-t2*) in non-dormant seeds (Piskurewicz *et al*., 2009). Curiously, exogenously applied ABA has been shown to repress endosperm rupture but not seed coat rupture (Müller *et al*., 2006; Linkies *et al*., 2009; Piskurewicz *et al*., 2009). However, under low GA conditions induced by paclobutrazol, *aba1*-6 mutant seeds exhibit seed coat rupture, suggesting that endogenous ABA synthesis is

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still somehow required in the repression of both endosperm and seed coat rupture (De Giorgi *et al*., 2015).

ABA-INSENSITIVE 3 (ABI3), ABI4 and ABI5 encode B3-, AP2- and bZIP-type transcription factors, respectively, which are important for the ABA responses during seed maturation, seed germination and seedling establishment. ABI3 is a seed- specific regulator that plays a crucial role in all three of these seed stages (Giraudat *et al*., 1992; Parcy *et al*., 1997; Nambara *et al*., 2000; Finkelstein *et al*., 2002; Lopez- Molina *et al*., 2002; Delmas *et al*., 2013). ABI4 has been shown to play a role in controlling oil mobilization in the embryo of germinating seeds (Penfield *et al*., 2006b) while ABI5 has been shown to control germination downstream of ABI3 by regulating GA and ABA responses (Lopez-Molina *et al*., 2001, 2002; Lee *et al*., 2012).

Analysing the expression pattern of ABI transcription factors during seed germination in the presence of ABA, we have previously observed that ABI3 is expressed ubiquitously throughout the seed, including the endosperm, while ABI4 is restricted to the embryo (Penfield *et al*., 2006b). Both ABI3 and ABI5 are expressed throughout the embryo but unlike ABI3, ABI5 expression is confined to the micropylar region of the endosperm through which the radicle emerges (Penfield *et al*., 2006b). This region of the endosperm plays an important role in the regulation of seed germination and in many species is characterized by a distinct gene expression pattern and cell wall composition compared with the lateral endosperm (Bewley, 1997).

Remarkably, GA biosynthesis pathway genes (*GA3ox1* and *GA3ox2*) can be traced to the cortex and endodermis cells in the hypocotyl transition zone that exhibits early expansion to complete germination, suggesting that GA triggers germination by specific cell expansion (Yamaguchi *et al*., 2001; Topham *et al*., 2017). Additionally, GA induces genes encoding cell-wall-modifying enzymes such as expansins (EXPAs), which constitute one of the four molecular mechanisms of cell wall loosening in plants (Cosgrove, 2005). Expansins are encoded by a multi-gene family and are very conserved among higher plants (Shcherban *et al*., 1995; Hutchison *et al*., 1999; Cosgrove, 2005). Expansins are thought to play multiple roles during the life cycle of higher plants, including in seed germination (Chen and Bradford, 2000; Chen *et al*., 2001; Yan *et al*., 2014; Marowa *et al*., 2016).

We have previously shown that cotyledon expansion precedes radicle protrusion during Arabidopsis seed germination and that this process is mediated by DELLA-protein growth repressors and ABA (Penfield *et al*., 2006a). Given that seed coat rupture requires expansion of the embryo, we set out to investigate the contribution of the ABI3, ABI4 and ABI5 transcription factors in the repression of seed coat rupture in *A. thaliana* seeds.

Materials and methods

Plant materials and growth conditions

All transgenic lines used in this study were described previously: *abi3-5* (*abi3*) (Ooms *et al*., 1993); *abi4-1* (*abi4*) (Penfield *et al*., 2006b); *abi5-7* (*abi5*) (Nambara *et al*., 2002); *ga1-3* (*ga1*) (Piskurewicz *et al*., 2009); and *ga1-3 rgl2-1 gai-t6 rga-t2* (*ga1 della3*) (Piskurewicz *et al*., 2009). *abi4* and *abi5* were in Columbia (Col) background, while *abi3*, *ga1* and *ga1 della3* were in Landsberg erecta (Ler). Plants were grown in a greenhouse supplemented with artificial light to give a photoperiod of 16 h light at a temperature of 20–22°C. Seeds were harvested when plants had stopped flowering and siliques had started to dehisce, and size sieved using a sieve with mesh size of 250 µm. All seeds used in this study were after-ripened, i.e. seeds had lost primary dormancy.

Germination assays

Sterilized seeds were plated on medium containing 0.9% (w/v) water agar. Plates were supplemented with ABA (Sigma-Aldrich) according to the germination condition examined. Percentage of seed coat rupture and radicle protrusion was scored after 5 days of imbibition. Nicking was performed in seeds after 1 h of imbibition using a needle to puncture the seed according to Fig. 1A. Between 50 and 100 seeds were used to check seed coat rupture and radicle emergence and repeated at least twice. Differences between results stated in the text are statistically significant as determined by Student's *t*-test ($P \leq 0.01$). Pictures of seeds were taken using a GXMXTL3 stereo microscope (GT Vision) coupled with a GXCAM-HICHROME Camera (GT Vision).

Real-time PCR gene expression assays

Total RNA was extracted from three biological replicates of frozen seed tissue (200 nicked seeds were used for each replicate) as described previously (Vaistij *et al*., 2013). cDNA synthesis was performed using standard methods. qPCR was performed using iQ SYBR Green Supermix and the MyiQ Real-Time PCR detection system (Bio-Rad) according to the manufacturer's instructions. The primer sequences used in this study are described in Supplementary Table 1, and expression of UBQ11 was used for normalization.

Results and Discussion

Exogenous ABA blocks seed coat rupture of nicked seeds

Nicking is a common procedure used in both dormant and GA-deficient seeds to promote germination. In Arabidopsis, nicking disrupts the integrity of both the seed coat and the endosperm (Fig. 1A). In non-dormant seeds, as expected, disruption of the endosperm did not affect the appearance of radicle protrusion (Fig. 1B, bottom panels). This finding is consistent with the fact that the role of the endosperm is to synthesize and continuously release ABA towards the embryo to block germination of dormant seeds (Lee *et al*., 2010; Kang *et al*., 2015). However, because nicking disrupts the integrity of the seed coat, we questioned whether or not nicking would also affect the appearance of seed coat rupture during germination, i.e. display radicle protrusion without prior seed coat rupture. For the purpose of this work, we only considered as non-artificial seed coat rupture (not promoted by puncturing) those longitudinal openings that were at least a third the size of the seeds. Nicking was performed in non-dormant Col seeds after 1 h of imbibition using a needle to puncture the chalazal region in order to create a continuum between the embryo and external medium (Fig. 1A). Intact Col seeds showed seed coat and endosperm rupture after 29 and 42 h of imbibition, respectively (Fig. 1B, top panels), while Col nicked seeds showed seed coat and endosperm rupture after 24 and 29 h of imbibition, respectively (Fig. 1B, bottom panels). Thus seed coat nicking does not affect the appearance of seed coat rupture *per se*, but it does decrease the post-imbibition period to both seed coat rupture and radicle protrusion compared with intact seeds.

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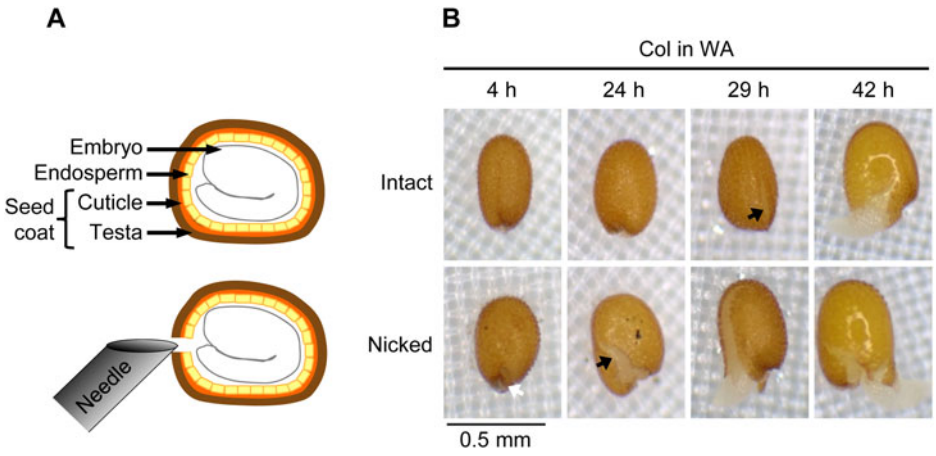

Fig. 1. Effect of nicking on the two-step germination of Arabidopsis Col seeds. (A) Scheme of a mature Arabidopsis seed showing the different tissues (on top) and the nicking technique applied to a seed using a 0.3 mm ø needle. Nicking (white arrow) was performed after 1 h of imbibition. (B) Images show intact and nicked non-dormant Col seeds that were imbibed in water agar (WA) plates. Black arrow: testa rupture. Scale bar is 0.5 mm.

In tetrazolium uptake assays, several reports have shown that defects in the seed coat properties are associated with less dormancy, more permeability to tetrazolium salts and more sensibility to exogenous ABA (Debeaujon *et al.*, 2000; Beisson *et al.*, 2007; Vishwanath *et al.*, 2013; MacGregor *et al.*, 2015). De Giorgi *et al.* (2015) reported a thick cutin-containing cuticular layer inside the seed coat which surrounds the whole endosperm of Arabidopsis seeds. These authors also showed that toluidine blue staining of embryos only occurs when this cuticle is disrupted. Hence, we hypothesized that the embryo is inaccessible to exogenous ABA and this is why ABA treatment does not block seed coat rupture in non-dormant seeds. To investigate this, we combined seed coat nicking and exogenous ABA treatment for 120 h after imbibition. Consistent with previous work (Müller *et al.*, 2006), 10 µM ABA-treated intact wild-type seeds all showed seed coat rupture, but no radicle protrusion (Fig. 2B, C). Following seed coat nicking, 10 µM ABA efficiently blocked seed coat rupture of both Ler and Col seeds (Fig. 2B,C,E,F). At 5 µM ABA nicked Col seeds showed a decreased frequency of seed coat rupture (Supplementary Fig. 1). These results demonstrate that seed coat nicking results in seed coat rupture becoming sensitive to exogenous ABA, presumably because the nicking results in the ABA accessing the embryo and blocking expansion. In the case of exogenous ABA, we propose that the cuticle prevents ABA uptake until the embryo expands and causes seed coat rupture, after which ABA then is taken up and blocks any further expansion of the radicle and endosperm rupture. Nicking of the seed coat will disrupt the cuticle, allowing immediate uptake of ABA and blockage of embryo expansion seed coat rupture and radicle emergence. This explanation is consistent with our previous demonstration that cotyledon expansion is necessary for seed coat rupture (Penfield *et al.*, 2006a).

ABI5 represses seed coat rupture of nicked seeds in the presence of exogenous ABA

Previous studies have shown that DELLA loss of function mutations lead to an induction of seed coat and endosperm rupture in Arabidopsis under low GA conditions (Cao *et al.*, 2005; Piskurewicz *et al.*, 2009). We previously reported that GA and the DELLA proteins regulate cotyledon expansion prior to endosperm rupture (Penfield *et al.*, 2006a). Consistent with these reports, we found that the *della3* mutant rescues the *ga1* phenotype by showing seed coat rupture but not radicle emergence through the endosperm of intact seeds treated with ABA (Fig. 2A). However, when nicked, only a small percentage of the *ga1 della3* seeds showed seed coat rupture and about 30% showed radicle protrusion without prior visible seed coat rupture in the presence of ABA, while *ga1* seeds remained quiescent (Fig. 2A, D). The fact that DELLA proteins, mainly RGL2, promote ABA synthesis (Lee *et al.*, 2010) could explain the leaky seed coat rupture and radicle protrusion observed in the *ga1 della3* nicked seeds. Taken together, these results suggest that DELLA proteins are at least partly involved in the ABA-dependent repression of seed coat rupture and radicle protrusion during germination.

To establish if the ABA-insensitive transcription factors are involved in the response of nicked seeds to exogenous ABA, we used loss of function *abi3*, *abi4* and *abi5* mutants. Consistent with previous reports (Ooms *et al.*, 1993; Finkelstein, 1994; Bies-Etheve *et al.*, 1999), we found that intact *abi3*, *abi4* and *abi5* seeds are insensitive to exogenous application of ABA in comparison with their respective wild-types (Fig. 2B,C). ABA-insensitive mutants *abi1-1*, *abi2-1* and *abi3-4* show a large cotyledon phenotype compared with their corresponding wild-types (Penfield *et al.*, 2006a). We did not observe seed coat rupture in either intact or nicked seeds of *abi3* in the presence of exogenous ABA, although seeds clearly showed radicle protrusion (Fig. 2B,E). ABI3 is a critical component of the network involved in many processes during seed development including the positive regulation of FUSCA3, a transcriptional factor that bears similarity to the B3 region of the ABI3-like proteins, which directly represses TRANSPARENT TESTA GLABRA1 (TTG1) (Tsuchiya *et al.*, 2004; Delmas *et al.*, 2013; Chen *et al.*, 2015). TTG1 also causes the transparent testa phenotypes, which includes deficiency in pigment accumulation in the seed coat (Debeaujon *et al.*, 2000; Sagasser *et al.*, 2002; Chen *et al.*, 2015). It is possible that altered composition of *abi3* seed coats renders them more resistant to rupture compared with wild-type when the radicle protrudes.

Nicked seeds of *abi4* do not show seed coat rupture in the presence of exogenous ABA (Fig. 2C,F), which is consistent with our previous findings that the events leading to germination in the endosperm of *abi4* seeds are completely blocked under concentrations of PAC or ABA inhibitory to wild-type seeds (Penfield *et al.*, 2006b). Bossi *et al.* (2009) demonstrated that ABI4 acts as a transcriptional activator of the expression of ABI5 through direct binding to CE1-like *cis*-acting elements in the promoter of ABI5; while Lopez-Molina *et al.* (2002) have shown that ABI5 acts downstream of ABI3 in order to repress germination in the presence of ABA. Thus, it is possible that, in the *abi4* mutant

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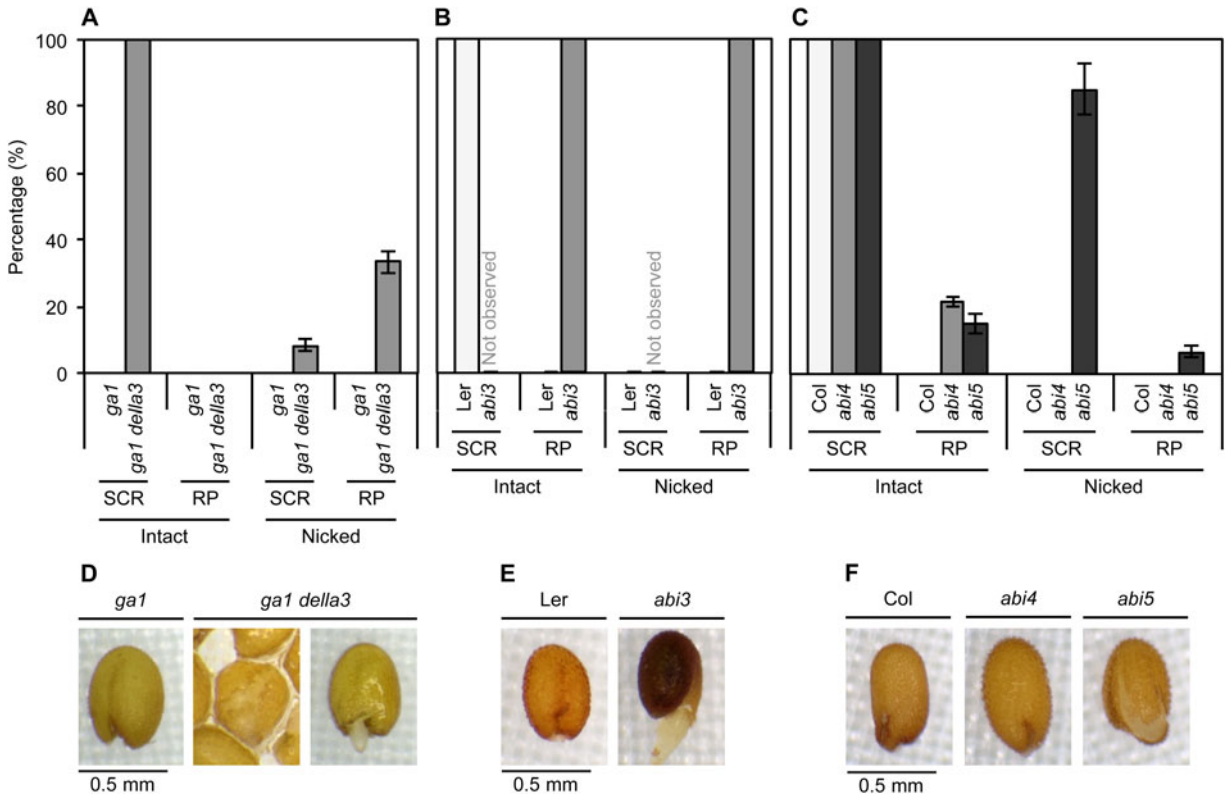

Fig. 2. Effect of ABA on the appearance of testa rupture in nicked seeds. (A–C) Percentage of seed coat rupture (SCR) and radicle protrusion (RP) in intact and nicked ga1, ga1 della3, Ler, abi3, Col, abi4 and abi5 seeds that were imbibed in water agar (WA) plates supplemented with 10 μM ABA for 120 h. Nicking was performed according to Fig. 1A. (D–F) Representative images showing ga1, ga1 della3, Ler, abi3, Col, abi4 and abi5 seeds 120 h after imbibition in WA plates supplemented with 10 μM ABA. Scale bar is 0.5 mm.

background, an early exposure to ABA promoted by nicking blocks seed coat rupture via the ABI3/ABI5 pathway described by Lopez-Molina *et al.* (2002).

As expected, *abi5* nicked seeds are insensitive to exogenous ABA showing more than 80% seed coat rupture (Fig. 2C,F). In contrast to these findings Piskurewicz *et al.* (2008, 2009) indicate that ABI5 is not involved in the repression of seed coat rupture under low GA conditions. However, this rather contradictory result may be due to the fact that GA is required to promote seed coat rupture.

ABI5 regulates expression of EXPANSINs in nicked seeds treated with ABA

Piskurewicz and others (2008, 2009) reported that RGL2, RGA and GAI are responsible for repression of seed coat rupture under low GA conditions, while Lee *et al.* (2012) have shown that ABI5 directly regulates the expression of *RGL2*, *RGA* and *GAI* under FR conditions. However, we did not observe any differences in the transcript levels of *RGL2*, *RGA* or *GAI* between Col and *abi5* nicked seeds in the presence of ABA (Fig. 3).

A recent study showed that seeds of an endosperm-specific expansin mutant (*exp2*) are delayed in germination in comparison with the wild-type (Yan *et al.*, 2014). In addition to this, using the β-glucuronidase (GUS) assay with a pAtEXP2:GUS construct these authors observed GUS staining in the radicle prior to germination. In an earlier study, a tomato expansin, LeEXPA4, was found to be involved in the control of germination by contributing to cell wall loosening in the micropylar endosperm (Chen and Bradford, 2000). Interestingly, in the presence of ABA, *ABI5* expression is limited to the micropylar region of the endosperm, but also expressed in the embryo (Penfield *et al.*, 2006b), which led us to speculate that ABI5 is involved in the repression of genes associated with cell wall loosening in the micropylar region of the endosperm through which the radicle must emerge for germination to occur.

Publically available transcriptomic data (vseed.nottingham.ac.uk) was used to select seven expansin genes that are highly expressed in imbibed Arabidopsis seeds for comparative expression analysis in *abi5* and wild-type. Remarkably, *EXPANSIN 1* (*EXPA1*), *EXPA2*, *EXPA3*, *EXPA9* and *EXPA20* expression were all increased in *abi5* nicked seeds compared with Col in the presence of ABA, while no significant changes were observed in the expression of *EXPA8* and *EXPA10* (Fig. 3). These results support a role for ABI5 in repressing the expression of expansins which in turn could lead to decreased embryo expansion and failure of the seed coat to rupture in the presence of ABA. It will be interesting to establish if the same suite of expansin genes are altered in an ABI5-dependent manner in the micropylar region of the endosperm and in the embryo in response to ABA.

Conclusion

Combining the data presented here with the current understanding of how seed coat rupture is regulated, we propose that ABI5 plays a key role in integrating ABA and GA responses in addition to DELLA proteins to control embryo expansion. Previous reports have established that control of seed coat rupture involves

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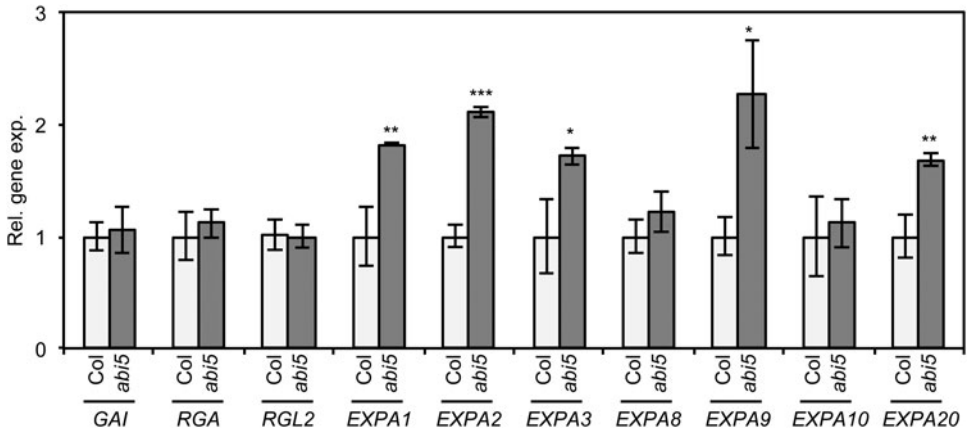

Fig. 3. Expression of DELLA and EXPANSIN genes in nicked Col and abi5 seeds in the presence of ABA. Transcript levels of GAI, RGA, RGL2, EXPA1, EXPA2, EXPA3, EXPA8, EXPA9, EXPA10 and EXPA20 genes, normalized to UBQ11 expression and expressed relative to Col, in 24 h ABA-treated Col and abi5 nicked seeds. Data are means ± SD of three biological replicates. Asterisks indicate statistically significant difference according to two-tailed Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

modulation of GA and ABA responses mediated by DELLA proteins, largely by RGL2 (Piskurewicz *et al.*, 2008, 2009; De Giorgi *et al.*, 2015). In the present work we have shown that, when exogenous ABA is readily available to the embryo as a consequence of nicking, ABI5 is required to repress seed coat rupture. Our data further indicate that ABI5 may act via repression of EXPANSIN genes, which are associated with cell wall loosening, thus affecting embryo expansion.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0960258519000059

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