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Original Article

Molecular mechanisms underlying the entrance in secondary dormancy of *Arabidopsis* seeds

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ABSTRACT

As seasons change, dormant seeds cycle through dormant states until the environmental conditions are favourable for seedling establishment. Dormancy cycle is widespread in the plant kingdom allowing the seeds to display primary and secondary dormancy. Several reports in the last decade have focused on understanding the molecular mechanisms of primary dormancy, but our knowledge regarding secondary dormancy is limited. Here, we studied secondary dormancy induced in *Arabidopsis thaliana* by incubating seeds at 25 °C in darkness for 4 d. By physiological, pharmacological, expression and genetics approaches, we demonstrate that (1) the entrance in secondary dormancy involves changes in the content and sensitivity to GA, but the content and sensitivity to ABA do not change, albeit ABA is required; (2) *RGL2* promotes the entrance in secondary dormancy through *ABI5* action; and (3) multivariate analysis with 18 geographical and environmental parameters of accession collection place suggests that temperature is an important variable influencing the induction of secondary dormancy in nature.

Key-words: ABA; GA; natural variation; primary dormancy; secondary dormancy.

INTRODUCTION

Seed dormancy is one of the most important adaptive traits in plants because it is a developmental checkpoint, allowing plants to regulate when and where they grow. Dormancy is usually defined as the failure of a viable seed to germinate in conditions otherwise favourable (Bewley 1997). Primary dormancy refers to the innate dormancy possessed by seeds when they are dispersed from the mother plant, while secondary dormancy refers to a dormant state that is induced in non-dormant seeds by conditions unfavourable for germination or re-induced in dormant seeds after a sufficiently low dormancy had been attained (Bewley 1997). The release from primary dormancy followed by a subsequent entrance into secondary dormancy leads to dormancy cycling pattern in nature.

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Dormancy cycling is a well-characterized trait in weed seed banks of agricultural lands (Benech-Arnold *et al.* 2000). Indeed, the persistence of a seed bank is not only related to the degree of primary dormancy but also influenced by the existing conditions that induce secondary dormancy leading to dormancy cycling in the population (Fenner 1995; Baskin and Baskin, 1998). The annual cycling of non-dormant and dormant states in the seeds is principally controlled by soil temperature and affected by soil water content (Bewley, 1997; Batlla and Benech-Arnold, 2006). Variations in dormancy are associated with changes in responsiveness to light and nitrate that act as factors that terminate dormancy and induce germination (Derkx and Karssen 1993, 1994; Botto *et al.* 1998). In fact, dormancy cycling involves repeated induction and termination of dormancy parallel to seasonal variations in soil temperatures. In this way, seeds avoid germination during short favourable conditions in the soil within the unfavourable season and germinate just prior to the favourable season for plant growth (Hilhorst 2007).

Dormancy research is focused on the induction and relief of primary dormancy with less attention paid to secondary dormancy (Finch-Savage and Leubner-Metzger 2006; Finkelstein *et al.* 2008; Holdsworth *et al.* 2008). To understand the molecular bases underlying the dormancy process, studies have adopted genetics and, recently, global expression approaches using model species such as *Arabidopsis thaliana* (Bentsink and Koornneef, 2002), Solanaceae (Auge *et al.* 2009) and cereals (Gubler *et al.* 2005). Dormancy release is regulated by a combination of environmental and hormonal signals with both synergistic and competing effects. The balance between abscisic acid (ABA) and gibberellin (GA) contents and sensitivity is a key regulator of the dormancy status of the seeds. In developing seeds, ABA promotes primary dormancy induction and later maintenance, whereas GA promotes the release of dormancy and germination. Environmental signals regulate the balance between ABA and GA by modifying the expression of metabolic enzymes, and also positive and negative regulators of both hormones, many of which are feedback regulated, that enhance or attenuate the dormancy levels (Finkelstein *et al.* 2008). By a transcriptomic approach, Cadman *et al.* (2006) identified substantial differences in gene expression between primary and secondary dormant seeds. Furthermore, the dormancy levels of buried seeds are tightly correlated with gene expression patterns following seasonal changes in soil temperature (Footitt *et al.* 2011, 2013). For

example, dormancy in Cape Verde Islands *Arabidopsis* seeds increases during winter when the expression of ABA synthesis (*NCED6*) and GA catabolism (*GA2ox2*) genes enhances ABA content, signalling and sensitivity. When dormancy declines in spring and summer, endogenous ABA decreases with the increase of ABA catabolism (*CYP707A2*) and GA synthesis (*GA3ox1*) together with the expression of negative ABA signalling components such as *ABI2* and *ABI4* (Footitt *et al.* 2011). *DELAY OF GERMINATION1* gene is relevant in secondary dormancy induction in response to temperature (Murphey *et al.* 2015), and its expression fluctuates during the year in buried seeds accompanying variations in secondary dormancy (Footitt *et al.* 2011).

Although our knowledge on the mechanisms underlying dormancy has increased in recent years, we do not have sufficient information about the mechanistic differences between primary and secondary dormancy (Hilhorst 2007). Here, we examined the mechanism of the entrance in secondary dormancy for *A. thaliana* seeds. We exposed imbibed and non-dormant seeds to darkness at a constant temperature, a condition that induces the entrance in secondary dormancy (Cadman *et al.* 2006). By physiological, pharmacological, expression and genetics approaches, we demonstrated that (1) the entrance in secondary dormancy involves changes in the content and sensitivity to GA, but the content and sensitivity to ABA do not change, albeit ABA is necessary; (2) *RGL2* promotes the entrance in secondary dormancy through *ABI5* action; and (3) in spite of the importance of temperature for secondary dormancy, we did not find a significant correlation between the latitudinal gradient of the accession collection place and the capacity to acquire secondary dormancy.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana plants were grown in a greenhouse at the Faculty of Agronomy, University of Buenos Aires (34°35′S, 58°29′W). Plants were cultivated during winter season with natural radiation (PAR = 350 μmol m^{-2} s^{-1}), photoperiod between 10 and 11 h, and average temperature of 21 ± 2 °C. Plants were grown together, and their mature seeds were harvested at the same time to avoid differences in post-maturation that can affect germination. Seeds of each genotype were harvested as a single bulk consisted of at least four plants. Seeds were stored in open tubes inside a closed box and maintained in darkness with silica gel at 25 °C.

Seeds of DELLA mutants in L*er* background were a gift from Dr Harberd, and seeds of 35S::HA–*ABI5* in *RGL2* were a gift from Dr López-Molina and described in Piskurewicz *et al.* 2008. Seeds of the accessions were obtained from the Arabidopsis Biological Resource Center.

Germination conditions and light treatments

Samples of 20 seeds per genotype were sown in clear plastic boxes, each containing 5 mL of 0.8% (w/v) agar in demineralized water. To establish a minimum and equal photoequilibrium, seeds were imbibed for 2 h in darkness and then irradiated for 30 min with a saturated far-red (FR) light, 700-800m pulse (Pfr/P = 0.03, 42 μmol m^{-2} s^{-1}). Then the seeds were incubated at 25 °C in darkness for 1–4, 7 and 8 d to induce secondary dormancy before a 30 min saturated R pulse (Pfr/P = 0.87, 35 μmol m^{-2} s^{-1}). After the light treatment, the boxes containing the seeds were wrapped again in black plastic bags and incubated at 25 °C for 3 d before germination was determined. The criterion for germination was the emergence of the radicle.

For the germination assays where hormones were used, seeds were sown in clear plastic boxes, each containing filter papers imbibed with GA or ABA (0.1, 1, 10 and 100 μM) with paclobutrazol (50 μM, Sigma-Aldrich, Steinheim, Germany) or fluridone (100 μM, Sigma-Aldrich) until the end of the experiment, respectively. We have previously performed germination assays and plotted calibration curves to determine the minimum concentration of paclobutrazol required to completely inhibit or fluridone required for maximum promotion of germination of seeds (Supporting Information Fig. S1).

Quantification of gibberellin and abscisic acid levels

Samples for GA and ABA quantification were collected 5 h after irradiation with the R pulse, along with the corresponding dark control. For quantifying ABA and GA4, 100 mg of seeds were ground and incubated at 4 °C for 12 h in 2 mL of isopropanol:acetic acid (99:1). After 5 min of centrifugation at 13 000 *g* at 4 °C, the supernatant was removed. The pellet was re-extracted with 1 mL of extraction solution for 2 h, and the supernatant was combined with that from the first extraction. This was dried and re-suspended in 50 μL of methanol. Solutions of 50 ng of d_2-GA1, d_2-GA4 and d_6-ABA were added as internal standards. Two microlitres of samples were analysed using ultra-performance liquid chromatography–mass spectrometry, according to the protocol described in Dave *et al.* (2011).

Gene expression analysis by quantitative RT-PCR

Samples of seeds (28 mg) were sown in clear plastic boxes, each containing 3 mL of 0.8% (w/v) agar in de-mineralized water. Three samples were harvested 5 h after the R pulse with the corresponding dark control and stored at −70 °C. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), adding 4% of polyvinylpyrrolidone (PVP) to the extraction buffer. cDNA derived from the extracted RNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and oligo-dT primers. The synthesized cDNAs were amplified with FastStart Universal SYBR Green Master (Roche, Madison, WI, USA) using the 7500 real-time PCR system cycler (Applied Biosystems, Foster City, CA, USA). *UBC2* gene was used as a normalization control (Czechowski *et al.* 2005). The specific primers used are described in Supporting Information Table S1.

Natural variation experiment

We evaluated the entrance in secondary dormancy of 230 accessions. The seeds used in the experiment were harvested from plants cultivated in white light chamber (16/8 h light/dark at 22 °C). The seeds were conserved in darkness at ambient temperature for 6 months until the experiment was performed. To homogenize germination, the seeds were incubated at 5 °C for 7 d prior to the treatments at 25 °C. Then the seeds were incubated in darkness at 25 °C for 1 or 4 d to evaluate the entrance in secondary dormancy (non-dormant and dormant seeds, respectively). Accessions with poor germination percentages (below 50% at 1 d of incubation in darkness) were excluded from the analysis. Finally, we used germination data of 135 accessions (Supporting Information Table S2). The experiment was repeated twice using four replicates of 20 seeds for each genotype. Because germination is dependent on the genetic background, the percentage of the entrance in secondary dormancy (%) was standardized by estimating the difference of germination between non-dormant and dormant conditions relative to the average value of germination of non-dormant seeds. Geographic and climatic data for each accession were obtained from WorldClim-Global Climate Data (http://www.worldclim.org/). We included nine temperature (annual mean temperature, temperature annual range, mean diurnal range, maximum of the warmest month, minimum of the coldest month and seasonal wettest, driest, warmest and coldest temperatures) and seven precipitation (annual precipitation, precipitation of the wettest and driest months and precipitations of the wettest, driest, warmest and coldest seasons) parameters. A principal component analysis (PCA) was conducted to represent the complexity of the data matrix in two principal axes. Percentage of the entrance in secondary dormancy of each accession was included in the analysis as the dependent variable, and geographical and environmental parameters were introduced as classification factors. The analyses were performed using INFOSTAT software [InfoStat/Professional version 1.1, Universidad Nacional de Córdoba, Córdoba, Argentina (http://www.infostat.com.ar/)].

Statistics and data analysis

Angular transformation of the percentage of germination was used when needed to meet the assumptions of the analysis. For hormone quantification and gene expression experiments, data were analysed using factorial analysis of variance, with time of incubation in darkness and light as independent factors. Differences between means were tested using Bonferroni post-test only when the relevant interaction terms were significant in the analysis of variance. The analyses were carried out with the GraphPad Prism Software version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

The entrance in secondary dormancy is induced by imbibition at 25 °C in darkness

Wild-type seeds were sown in water and incubated in darkness at 25 °C. The incubation time before the seeds were irradiated with an R pulse varied between 1 and 8 d. The R pulse induced 100% of germination when given after 1 d of dark incubation (Fig. 1). Larger periods in darkness at 25 °C induced the progressive entrance in secondary dormancy, and germination of R pulse-treated seeds was only 40% after 4 d and less than 10% after 7 d in darkness, reaching the same percentage as dark controls (Fig. 1). These results demonstrated that seeds acquired secondary dormancy during the incubation in darkness at 25 °C, losing their capacity to respond to an R pulse.

The entrance in secondary dormancy changes gibberellin but not abscisic acid sensitivity

To evaluate the role played by ABA and GA in the entrance in secondary dormancy, we analysed the germination of R pulse-treated seeds after incubation in darkness for 1 or 4 d. In this experiment, after incubation in darkness for 1 d, the germination of the R pulse-treated seeds was very high, while incubation in darkness for 4 d strongly reduced the response to the R pulse (91 versus 14%, respectively, Fig. 2a). The addition of GA4 doses in the incubation medium prevented the entrance in secondary dormancy in non-dormant seeds. The response to GA4 doses was biphasic in dormant seeds. Seed germination was partially promoted reaching up 60% by 0.1 μM of GA4 and fully promoted by GA4 doses higher than 10 μM (Fig. 2a).

To avoid the effects of endogenous GA, we incubated the seeds in a medium containing GA4 and paclobutrazol, which inhibits endogenous GA biosynthesis (Fig. 2b). In this experiment, non-dormant seeds germinated with increasing GA4 doses between 0 and 10 μM, while dormant seeds needed GA4 doses higher than 1 μM, indicating that the entrance in secondary dormancy induced changes in GA sensitivity (Fig. 2b).

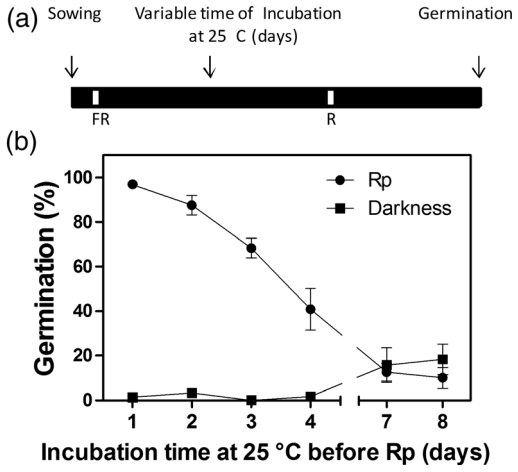

Figure 1. The entrance of *Arabidopsis thaliana* seeds into secondary dormancy. (a) Experimental protocol for the entrance in secondary dormancy. Seeds were sown for 2 h in darkness and then irradiated with an FR pulse. Seeds were then incubated at 25 °C in darkness for variable time and finally irradiated with an R pulse (Rp). Germination was counted after 3 d at 25 °C. (b) Germination of Col-0 seeds imbibed at 25 °C in darkness for 1–4, 7 and 8 d before an R pulse. A darkness control treatment without the R pulse is shown. Each point represents mean ± SE ($n = 6$).

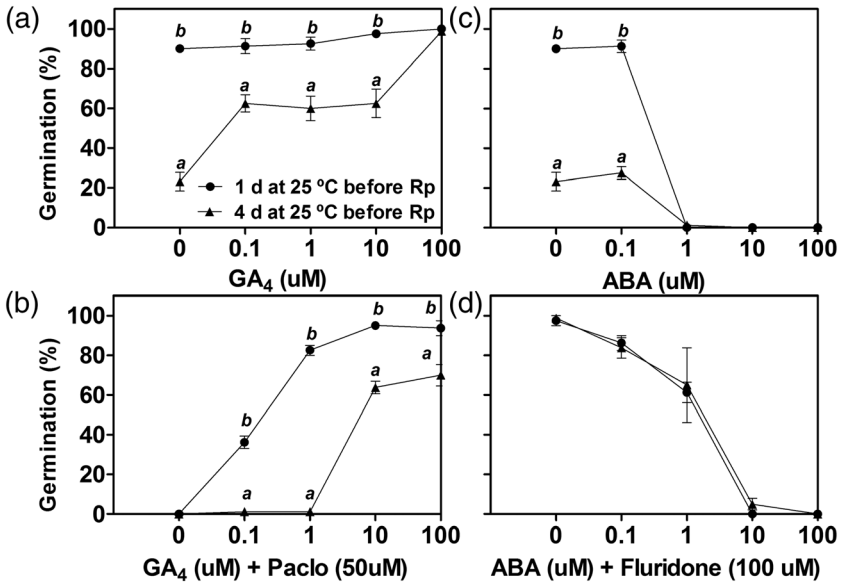

Figure 2. The entrance in secondary dormancy alters the GA but not the ABA sensitivity in Col-0 seeds. Each point of percentage of germination (%) represents mean ± SE ($n = 4$). Only significant differences between means at the same hormone concentration are shown with different letters ($P = 0.05$ by *t*-test). Germination (%) data were transformed by angular transformation to meet the assumptions of the statistical analysis. ABA, abscisic acid; GA, gibberellin; Rp, R pulse.

The addition of ABA inhibited germination in non-dormant and dormant seeds. Without ABA, non-dormant seeds germinated 89%, and dormant seeds germinated 32% after an R pulse (Fig. 2c). The germination of seeds was completely inhibited by 1 μM ABA, irrespective of the dormancy state (Fig. 2c). To study the sensitivity of the seeds to ABA, we incubated the seeds in a medium with ABA and fluridone, which inhibits endogenous ABA synthesis (Supporting Information Fig. S1). The addition of fluridone in a medium without ABA promoted the germination of seeds, irrespective of the state of dormancy (Fig. 2d). Increasing the concentration of ABA in the medium reduced the germination of non-dormant and dormant seeds in an identical manner, where 10 μM ABA led to complete inhibition of germination. On the other hand, the promotion of germination of dormant seeds by fluridone indicates that ABA synthesis is necessary for the induction of secondary dormancy. Thus, it appears that the entrance in secondary dormancy is regulated by the balance between GA and ABA, but it is not influenced by the ABA sensitivity of seeds.

The entrance in secondary dormancy alters gibberellin but not abscisic acid content

Taking into account the influences of GA and ABA on the entrance in secondary dormancy, we asked whether the levels of these hormones change differentially in non-dormant and dormant seeds maintained in darkness or irradiated by a light pulse. Hormone contents were analysed in seeds incubated in darkness for 1 or 4 d before being irradiated or non-irradiated with an R pulse (Fig. 3). Non-dormant seeds showed higher content of GA than dormant seeds (90 versus 50 ng mg^{-1} dry weight, P time < 0.01, Fig. 3a), and the light factor did not alter

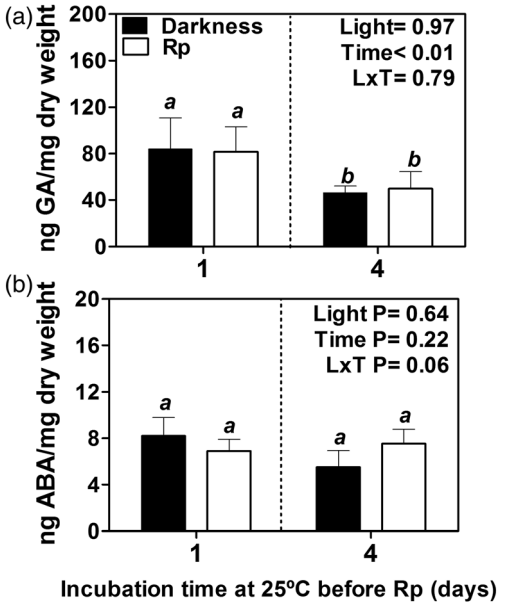

Figure 3. The entrance in secondary dormancy alters the GA but not the ABA content independently of light in Col-0 seeds. GA (a) and ABA (b) contents in the seeds are shown. Each bar represents mean ± SE ($n = 3$). The *P*-value of each factor in the two-way analysis of variance is shown in the top right side of the figures. Significant differences between means are shown by Bonferroni post-test. ABA, abscisic acid; GA, gibberellin; Rp, R pulse.

the GA content in the seeds (P light = 0.97, Fig. 3a). In contrast, ABA levels in the seeds were not affected neither by the dormancy state nor by light (P time = 0.22 and P light = 0.64, Fig. 3b). These results confirm that the entrance in secondary dormancy involves reduction of GA levels but not changes in ABA levels.

RGL2 promotes the entrance in secondary dormancy through *ABI5* action

We studied the expression during entrance in secondary dormancy of some of the genes known to be involved in dormancy and germination. We evaluated biosynthesis (*GA3ox1* and *NCED9*) and catabolic (*GA2ox2* and *CYP707A2*) genes associated with GA and ABA, respectively (Fig. 4). We observed a general pattern of reduction of gene expression associated with the

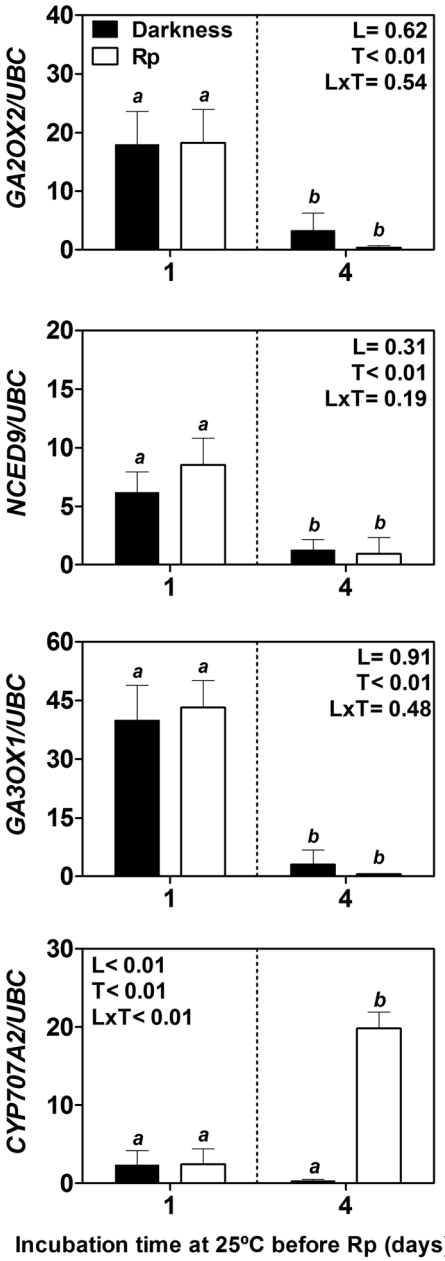

Figure 4. The entrance in secondary dormancy reduces the expression of GA and abscisic acid metabolic genes in Col-0 seeds. Biosynthesis (*GA3ox1* and *NCED9*) and catabolic (*GA2ox2* and *CY707A2*) genes are shown. Each bar represents mean ± SE ($n = 3$). The *P*-value of each factor in the two-way analysis of variance is shown in each graph. Significant differences between means are shown by Bonferroni post-test. GA, gibberellin; Rp, R pulse.

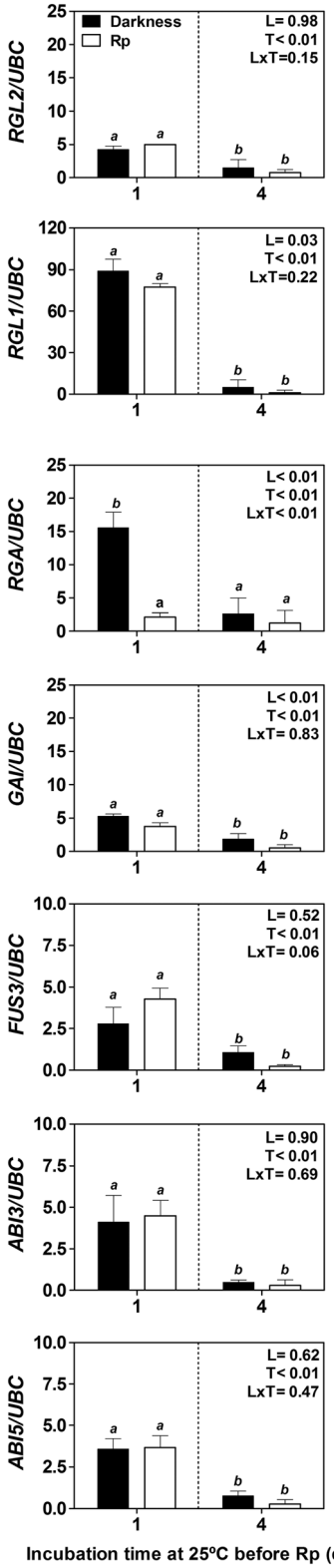

Figure 5. The entrance in secondary dormancy reduces the expression of GA and ABA sensitivity genes in Col-0 seeds. Signalling GA (*RGA*, *GAI*, *RGL2* and *RGL1*) and ABA (*FUSCA3*, *ABI3* and *ABI5*) genes are shown. Each bar represents mean ± SE ($n = 3$). The *P*-value of each factor of the two-way analysis of variance is shown in each graph. Significant differences between means are shown by Bonferroni post-test. ABA, abscisic acid; GA, gibberellin; Rp, R pulse.

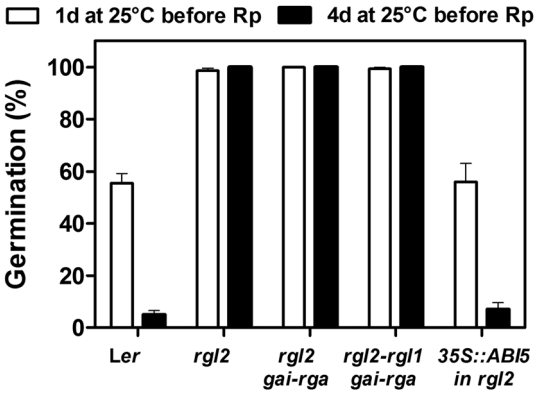

Figure 6. The entrance in secondary dormancy requires *RGL2* through the *ABI5* activity in L*er* seeds. Germination of single, triple and quadruple DELLA mutants and the 35S::HA–*ABI5* in *RGL2* mutant seeds exposed to an R pulse (Rp) after imbibition for 1 or 4 d in darkness at 25 °C. Each bar represents mean ± SE ($n \geq 6$).

entrance in secondary dormancy (P time < 0.01, Figs 4 & 5). Light factor did not have significant effects, except for *CYP707A2* gene, in which the R pulse in dormant seeds dramatically promoted its expression (P light × time < 0.01, Fig. 4). Furthermore, we analysed the expression of four DELLAs and three ABA signalling genes (Fig. 5). Irradiation with an R pulse significantly reduced the expression of *RGA* in non-dormant seeds (P light × time < 0.01, Fig. 5).

To have a better understanding of the functional roles played by GA-related and ABA-related factors in the entrance in secondary dormancy, we studied the germination of different mutants in our experimental conditions (Fig. 6). The germination of DELLA mutants was evaluated in L*er* background. Wild-type seeds germinated 55 and 5% in response to an R pulse after incubation for 1 and 4 d in darkness, respectively, indicating that secondary dormancy was fully induced (Fig. 6). The absence of *RGL2* in single, triple and quadruple DELLA mutants impeded the seeds to enter into secondary dormancy, suggesting a prominent function of *RGL2* in the control of secondary dormancy (Fig. 6). Based on previous evidence, demonstrating that *RGL2* regulates *ABI5* activity and ABA biosynthesis during seed germination (Ibarra *et al.* 2013; Piskurewicz *et al.* 2008), we evaluated the function of *ABI5* in the context of the *RGL2* background in our experimental conditions. Interestingly, the over-expression of *ABI5* recovered the entrance in secondary dormancy of *RGL2* seeds (Fig. 6). These results suggest that *ABI5* is functional in the entrance of secondary dormancy acting downstream of *RGL2*.

The entrance in secondary dormancy is not associated with a latitudinal gradient

We evaluated the entrance in secondary dormancy of seeds from 135 accessions collected from various locations around the world (Supporting Information Table S2). To homogenize germination, the seeds were incubated at 5 °C for 7 d before the incubation in darkness to induce secondary dormancy. A wide germination variation between accessions was found for seeds incubated in darkness for 1 or 4 d at 25 °C (inserted graph in Fig. 7). The entrance in secondary dormancy ranged between 0 and 98% (Supporting Information Fig. S2), independently of the place of origin (Supporting Information Fig. S3). We estimated the Pearson correlation index between the entrance in secondary dormancy and location (latitude and longitude)

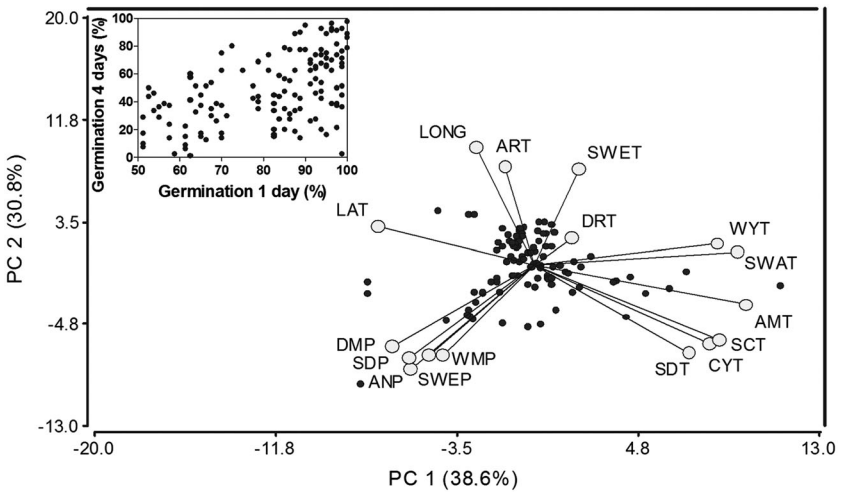

Figure 7. Principal component analysis (PCA) for the entrance in secondary dormancy (%) of 135 accessions and two geographical parameters (latitude, LAT; longitude, LONG), nine temperature parameters (annual mean temperature, AMT; annual range temperature, ART; mean diurnal range, DRT; maximum of the warmest month, WYT; minimum of the coldest month, CYT; and seasonal wettest, driest, warmest and coldest temperatures, SWET, SDT, SWAT and SCT) and seven precipitation parameters (annual precipitation, ANP; precipitation of the wettest and driest months, WMP and DMP; and precipitations of the wettest, driest, warmest and coldest seasons, SWEP, SDP, SWAP and SCP) from the collection place of accessions. PCA was conducted to reduce the complexity of the data matrix in two eigenvectors. All the measured variables for each genotype were included in the analysis as independent variables and the entrance in secondary dormancy (%) as a dependent factor. In the inserted graph, the correlation between germination after incubation of seeds for 1 or 4 d before irradiation with a saturated R pulse is shown. The black points represent the average response for each accession ($n = 3$).

Table 1. Pearson correlation coefficient (r), R^2 and P-value between entrance in secondary dormancy (%) and location (latitude and longitude), germination at 1 or 4 d after R pulse and nine temperature and seven precipitation variables (for reference, see Fig. 7)

Variable	Correlation index (Pearson, r)	R^2	P-value	Signif.
Lat	−0.068	0.004	0.432	ns
Long	−0.092	0.008	0.292	ns
Germ. 1 d	−0.237	0.056	0.006	**
Germ. 4 d	−0.931	0.867	<0.0001	***
AMT	0.132	0.017	0.128	ns
DRT	0.056	0.003	0.520	ns
WYT	0.105	0.011	0.225	ns
CYT	0.117	0.014	0.175	ns
ART	−0.032	0.001	0.711	ns
SWET	0.136	0.018	0.117	ns
SDT	−0.029	0.001	0.733	ns
SWAT	0.117	0.014	0.176	ns
SCT	0.106	0.011	0.222	ns
ANP	−0.090	0.008	0.298	ns
WMP	−0.149	0.022	0.085	ns
DMP	−0.006	0.000	0.941	ns
SWEP	−0.145	0.021	0.092	ns
SDP	−0.023	0.001	0.789	ns
SWAP	−0.067	0.004	0.438	ns
SCP	−0.066	0.004	0.443	ns

Lat, latitude; Long, longitude; Germ., germination; AMT, annual mean temperature; DRT, mean diurnal range; WYT, maximum of the warmest month; CYT, minimum of the coldest month; ART, annual range temperature; SWET, seasonal wettest temperature; SDT, seasonal driest temperature; SWAT, seasonal warmest temperature; SCT, seasonal coldest temperature; ANP, annual precipitation; WMP, precipitation of the wettest month; DMP, precipitation of the driest month; SWEP, precipitation of the wettest season; SDP, precipitation of the driest season; SWAP, precipitation of the warmest season; SCP, precipitation of the coldest season; ns, not significant.
**P <0.01.
***P < 0.001.

and 16 environmental variables (nine for temperature and seven for precipitation). The output of the analysis suggests a multivariate contribution on the expression of secondary dormancy because neither of them showed a significant correlation index (Table 1). Then we proceeded to do a multivariate analysis of PCA including 18 geographical location and environmental parameters (Fig. 7). The first and the second dimensions of the analysis (PC1 and PC2) explained 38.6 and 30.8% of the data variability (Fig. 7). The first axis ordered the cases following a pattern defined by temperature on the right side and precipitation and latitude grouped together on the other side. The second axis ordered the cases by temperature and longitude variables grouped together on the upper side and other temperature and precipitation parameters appeared together on the bottom of the graph. These results suggest that temperature and precipitation variables of accession collection place influence the capacity to enter in secondary dormancy.

DISCUSSION

Whereas primary dormancy is acquired during seed maturation, imbibed non-dormant seeds exposed to unfavourable environmental conditions or lacking adequate light or nitrate conditions may enter a state of secondary dormancy (Finkelstein *et al.* 2008). Several treatments allow the entrance in secondary dormancy in seeds, like dark imbibition or skotodormancy (Bewley *et al.* 2013; Cadman *et al.* 2006), high temperatures or thermodormancy (Argyris *et al.* 2008; Leymarie *et al.* 2008; Chiu *et al.* 2012; Bewley *et al.* 2013; Huo *et al.* 2013; Lim *et al.* 2013), imbibition in cold (Cadman *et al.* 2006; Finch-Savage *et al.* 2007), hypoxia (Hoang *et al.* 2013) and osmotic stress (Fei *et al.* 2009). Although in the last decade, we have learnt more about the molecular mechanisms underlying primary dormancy, our knowledge on secondary dormancy is still fragmented. At present, little is known about the molecular mechanisms regulating the process of secondary dormancy and also about differences between the mechanisms that impose primary and secondary dormancy. In this work, we used physiological, genetic and pharmacological approaches to improve our understanding of the molecular bases of secondary dormancy when non-dormant *Arabidopsis* seeds were exposed to dark imbibition at 25 °C, a treatment that induces secondary dormancy. We found that (1) the entrance in secondary dormancy involves changes in the content and sensitivity of GA, but the content of ABA and sensitivity to ABA do not change, albeit ABA is necessary; (2) *RGL2* promotes the entrance in secondary dormancy through *ABI5* action; and (3) multivariate analysis with 18 geographical and environmental parameters of accession collection place suggests that temperature is an important, but not the unique, cue influencing the induction of secondary dormancy in nature.

In our experiments, secondary dormancy was induced in *A. thaliana* seeds by a dark imbibition at 25 °C for 4 d (Fig. 1 and Supporting Information Fig. S2). The entrance in secondary dormancy is tightly dependent on the accession (i.e. the inhibition of germination ranged between 0 and 98%; see Supporting Information Fig. S2). For Col-0 and L*er* control genotypes, the 4-day imbibition in darkness before irradiation inhibited seed germination between 60 and 90% depending on experimental date or population batch (Figs 1, 2 & 6). The entrance in secondary dormancy reduced GA but not ABA content and sensitivity of seeds (Figs 2 & 3). The induction of secondary dormancy also decreased the expression of metabolism and signalling genes, with the exception of *CYP707A2* and *RGA* DELLA genes, which expression was affected by light (Figs 4 & 5). The expression of *CYP707A2*, a gene important for ABA inactivation, was increased by an R pulse in dormant seeds imbibed in darkness for 4 d. Furthermore, *RGA* DELLA expression was dramatically reduced when non-dormant seeds imbibed in darkness for 1 d were irradiated by an R pulse. Physiological, hormonal and expression results suggest that, although the ABA content is similar in non-dormant and dormant seeds, the entrance in secondary dormancy is finely regulated by the balance between GA and ABA. Previous studies have demonstrated that 20 d of dark imbibition at 20 °C (Cadman *et al.*, 2006) or high temperatures at 32 °C (Chiu *et al.* 2012; Huo *et al.* 2013; Lim *et al.* 2013) promotes secondary dormancy altering ABA content and GA–ABA balance in *Arabidopsis* seeds (Cadman *et al.* 2006; Footitt *et al.* 2011). The increase in ABA content and sensitivity is

also a common feature with regard to the entrance in secondary dormancy of lettuce, barley and rapeseed seeds (Argyris *et al.* 2008; Leymarie *et al.* 2008; Fei *et al.* 2009; Hoang *et al.* 2013). Considering these evidences, we hypothesize that different mechanisms may impose secondary dormancy in non-dormant seeds depending on the type or intensity of unfavourable imbibition conditions. In other words, despite that both GA and ABA hormones regulate the entrance in secondary dormancy, we suggest that weak unfavourable conditions that impose secondary dormancy may initially affect GA signalling elements and later, when dormancy increases, ABA signalling might become relevant. There is evidence supporting the idea that different mechanisms may act to impose secondary dormancy. In barley seeds, secondary dormancy induced by 30 °C increased ABA content and sensitivity (Leymarie *et al.* 2008), but the induction of secondary dormancy under hypoxia at 15 °C was regulated to a larger extent by GA, principally by modifications in metabolic genes, and to a lesser extent by ABA (Hoang *et al.* 2013). Furthermore, a natural functional variant of ABA biosynthesis *NCED4* gene is responsible for thermodormancy in lettuce seeds, where the heat stress rather than water stress is responsible for the induction of *NCED4* expression in leaves (Huo *et al.* 2013). Altogether, these results suggest that specific signalling mechanisms operate depending on the stress condition that imposes secondary dormancy.

By conducting experiments using mutant seeds, we found that *RGL2* imposed secondary dormancy in collaboration with *ABI5* acting downstream (Fig. 6). *RGL2* and *ABI5* have previously been shown to be involved in the control of germination by the opposite action of GA that enhances the destruction of *RGL2* and ABA that induces *ABI5* repression (Piskurewicz *et al.* 2008). This mechanism is also involved in the promotion of germination by the action of phytochrome A through a very low fluence of light (Ibarra *et al.* 2013). Recently, it was demonstrated that physical interaction between DELLAs (*RGA* and *GAI*), *ABI5* and ABI3 proteins targets SOMNUS (SOM) promoter and promotes its expression inhibiting germination at high temperatures, a treatment that induces secondary dormancy (Lim *et al.* 2013). However, future studies are needed to clarify if *RGL2* is also involved in protein interactions with ABI transcription factors or if it operates via another molecular mechanism. Furthermore, the *RGL2–ABI5* signalling pathway appears to be more complex than previously suspected. In fact, *ABI5* can also be activated by other transcription factors like HY5, which binds the *ABI5* promoter to inhibit germination and post-germination processes (Chen *et al.* 2008). Moreover, BBX21, a double B-Box protein, promotes germination under stress conditions inhibiting *ABI5* expression by interfering with HY5 binding to *ABI5* promoter (Xu *et al.* 2014).

Although no significant relationship between the latitudinal gradient of accession collection and the capacity of entrance in secondary dormancy was observed (Table 1), the PCA analysis indicates that the temperature regime of the accessions location of origin may be an important factor in the determination of the capacity to acquire secondary dormancy. Our results are in accordance with those reported by Debieu *et al.* (2013), who found a slight latitudinal gradient associated with primary dormancy but not with secondary dormancy for a wide set of European *Arabidopsis* accessions. In a smaller geographical area, Montesinos-Navarro *et al.* (2012) studied the association between primary and secondary dormancy and the genetic variation for 17 populations of *Arabidopsis* seeds collected in an altitudinal gradient of the northeast of Spain. In this study, the authors found strong primary dormancy and low secondary dormancy in mountainous populations exposed to cold winters and prolonged wet springs compared with coastal populations exposed to warm and dry summers and template winters (Montesinos-Navarro *et al.* 2012). These results suggest that primary dormancy is an adaptive response principally modulated by the temperature, whereas secondary dormancy is probably more affected by other factors that could explain the distribution of genetic variants in nature.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Calibration curves for seeds imbibed in different doses of paclobutrazol and fluridone. Each point of percentage of germination (%) represents mean ± SE ($n = 2$).

Figure S2. (a) The entrance in secondary dormancy for the 135 accessions ranked by increasing percentages of secondary dormancy. Entrance in secondary dormancy (%) was estimated from the germination data of Fig. 7A. Each bar represents mean ± SE ($n = 3$). (b) Entrance in secondary dormancy ranked by country. Only countries with more than 10 accessions were included in the analysis.

Figure S3. Correlation between the entrance in secondary dormancy (%) and germination of seeds incubated for 1 day (a) or 4 days (b) before irradiation with a Red pulse.

Table S1. Primers used in the study.

Table S2. Accessions used in the natural variation experiment. For each accession, the germination after 1 or 4 days of incubation in darkness at 25 °C and the entrance in secondary dormancy response as a percentage are indicated. The origin (location and country), and the temperature and precipitation parameters for each location used for PCA analysis are also indicated.