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ORIGINAL PAPER

Roles of abscisic acid and gibberellins in maintaining primary and secondary dormancy of Korean pine seeds

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Abstract Primary dormancy of seeds of Korean pine (*Pinus koraiensis* Sieb. et Zucc.) after dispersal in the autumn and the induction of secondary dormancy the first summer following seed dispersal limit the regeneration of mixed broadleaved Korean pine forests in Northeast China. This study was to determine how changes in the levels of abscisic acid (ABA) and gibberellic acid (GA) maintain primary and secondary dormancy of Korean pine seeds under germination conditions. We transferred seeds with one of five primary dormancy states or three secondary dormancy states to germination conditions and measured changes in the levels of ABA, GA_{1+3} (GA_1 and GA_3) and GA_{4+7} (GA_4 and GA_7) in the seed coat, megagametophyte and embryo during incubation. Seed coat ABA levels in primary dormant seeds (PDS) and ABA levels in various parts of secondary dormant seeds (SDS) gradually declined during incubation but were still higher than in seeds for which dormancy was progressively released. GA_{4+7} and GA_{1+3} levels in embryos greatly decreased 35% and 24%, respectively, during incubation of SDS, and thus, the ratio of ABA to GA_{4+7} in embryos and megagametophytes significantly increased. The ratio of ABA to GA_{1+3} in various parts of SDS increased slightly during incubation. In contrast, in seeds for which secondary dormancy was already released, GA_{4+7} and GA_{1+3} levels in the embryo, GA_{4+7}/ABA ratio in the embryo and seed coat, and the GA_{1+3}/ABA in the embryo and megagametophyte significantly increased during incubation. There was no trend in the changes in the levels of ABA, GA_{4+7} or GA_{1+3} in embryos and megagametophytes of PDS or the levels of GA_{4+7} or GA_{1+3} in megagametophytes of SDS during incubation. The results suggest that high ABA levels in the seed coat maintain primary dormancy of Korean pine seeds. Maintenance of secondary dormancy involves a reduction of GA_{4+7}, GA_{1+3}, GA_{4+7}/ABA, and GA_{1+3}/ABA and the retention of high ABA levels.

Keywords Abscisic acid · Gibberellic $acid_4$ and $acid_7$ · Gibberellic $acid_1$ and $acid_3$ · Korean pine · Primary dormancy · Secondary dormancy

Introduction

Seeds of many species in temperate regions undergo dormancy cycling in which dormancy is repeatedly released and induced (Baskin and Baskin 1998; Claessens 2012). Dormancy cycling is terminated when environmental conditions are favorable for germination (Claessens 2012). Primary dormancy is induced as the seed develops on the mother plant (Frey et al. 1999). As primary dormancy is gradually released, the range of conditions under which seeds can germinate progressively increase. At some point the seeds become nondormant and can germinate across the full range of suitable environments for the population (Baskin

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and Baskin 1998). If environmental conditions do not favor germination, nondormant seeds may gradually enter a new dormancy state, called secondary dormancy (Bewley et al. 2013). The time of induction of primary and secondary dormancy differs (Frey et al. 1999). For annual summer species in temperate climates, primary dormancy is released by low winter temperatures shortly after seed dispersal (Meulebrouck et al. 2010; Cao et al. 2014), whereas secondary dormancy is induced by high summer temperatures (Malavert et al. 2017; Gao et al. 2018).

The mixed broadleaved—Korean pine forest (MBKPF) is the climax vegetation from Northeast China to the Far East of Russia (Ishikawa et al. 1999). The distribution area of the MBKPF, however, has been decreasing in the past century due to large-scale industrial deforestation (Tian et al. 2009), and is in urgent need of restoration, which requires regeneration of Korean pine (*Pinus koraiensis* Sieb. et Zucc.), the dominant tree species in MBKPF, from seed. However, Korean pine seeds undergo dormancy cycling. When they are dispersed in the autumn, they have strong primary dormancy, classified as morphophysiological dormancy; i.e., the embryo is not only underdeveloped but also a physiological inhibitory component of dormancy (Qi et al. 1993). The physiological dormancy component of this morphophysiological dormancy (primary dormancy) of Korean pine seeds is released during the winter after their release. Any seeds that fail to germinate that spring then re-enter physiological dormancy (secondary dormancy) during the first summer after seed dispersal (Song et al. 2018). This secondary dormancy is released during the second winter after seed dispersal. This primary dormancy and secondary dormancy apparently lead to poor regeneration of Korean pine and prevent restoration of the MBKPF. Consequently, we need to explore the mechanisms of both primary and secondary dormancy that lead to release of seed dormancy in the context of promoting the restoration of MBKPF.

Abscisic acid (ABA) and gibberellic (GA) regulate seed dormancy (Koornneef et al. 2002). ABA is largely involved in the induction of seed primary dormancy and secondary dormancy (Leymarie et al. 2008). A high ABA level is associated with strong dormancy states (Finch-Savage and Leubner-Metzger 2006). GA is also implicated in the induction of primary dormancy (White et al. 2000; Ibarra et al. 2016). However, a reduction in ABA levels in primary dormant seeds is not sufficient to release dormancy (Chien et al. 1998). For example, after extensive rinsing, ABA levels in Korean pine seeds decrease to amounts similar to those present in moist, cold-stratified seeds, but only the moist, cold-stratified seeds can germinate (Tan et al. 1983; Gao et al. 1983). Moreover, the level of endogenous GA is not related to the primary dormancy of *Avena fatua*, because seeds in primary dormancy and those released from primary dormancy contain similar levels of gibberellin $acid_1$ (GA_1) (Metzger 1983). Additionally, there is no relationship between ABA and GA_3 levels and the germination potential of Korean pine seeds in different primary dormancy states (Song and Zhu 2016). Therefore, the seed dormancy state not only depends on the levels of ABA and/or GA, but also on a change in the patterns of ABA and/or GA levels when seeds are transferred to a germination-inductive temperature (Benech-Arnold et al. 2006). A higher level of ABA is also related to the maintenance of primary dormancy (Fidler et al. 2018). ABA in the embryo and megagametophyte, rather than gibberellic $acid_1$ and $acid_3$ (GA_{1+3}), gradually induce Korean pine seeds to enter secondary dormancy states (Song et al. 2018), but very little is known about how ABA regulates the maintenance of secondary dormancy. Little attention has been paid to tree seeds such as Korean pine that simultaneously have both primary dormancy and secondary dormancy at different times in the seed life cycle. Similarly, the regulation of GA during the maintenance of dormancy may also be involved in the decrease of GA when conditions are suitable for germination. However, no studies have yet verified this possibility. Such deficiencies in our knowledge on ABA and GA regulation of Korean pine seed dormancy has restricted efforts of restore the MBKPF.

Therefore, we analyzed the changes of ABA, GA_{1+3}, and GA_{4+7} (GA_4 and GA_7) in the seed coat, megagametophyte and embryo of seeds in different dormancy states during dormancy maintenance. The objective of the present study was to determine how ABA and GA regulate the maintenance of primary and secondary dormancy. Understanding the underlying maintenance mechanisms of primary and secondary dormancy will contribute to developing methods to release seed dormancy and thus promote the regeneration of Korean pine.

Materials and methods

Seed collection

In October 2014, fresh Korean pine seeds were obtained from a Korean pine plantation forest in Qingyuan Forest CERN, Chinese Academy of Sciences (CAS), Northeast China (4151.102N, 12456.543 E, 456-1116m a.s.l.). This region has a continental monsoon climate. During the spring (March, April, and May), the weather is warm and windy with a strong monsoon. In the summer (June, July, and August), the weather is humid and rainy. Winter (November, December, January, and February) is cold and dry. The mean annual air temperature is 4.7 °C, the maximum is 35.9 °C (July) and the minimum is 32 °C (January). The minimum annual precipitation is 700 mm, the maximum is 850 mm. More than 80% of the rainfall occurs in June, July and August. Freshly harvested seeds were stored at 20 °C to maintain their primary dormancy states and then used for the subsequent experiments.

Preparation of seeds with different primary dormancy states

In April 2015, freshly harvested seeds that had been stored at 20 °C were removed from storage and soaked in water for 7 days. Then, these imbibed seeds were mixed with moist sand and stored at 5 °C for 1, 2, 4 or 6 months of cold stratification. These four sampling times were selected based on the fact that (1) deep, intermediate and nondeep physiological dormant seeds require 34 months, 23 months, and 1 week of cold stratification, respectively, to release dormancy (Baskin and Baskin 2004; Kermode 2011), and (2) the duration of moist cold stratification that is applied in forestry practices to release Korean pine primary dormancy is approximately 6 months (Yao 1966). Germination percentage is used to determine the dormancy states (Hilhorst and Karssen 1992). We also analyzed seeds with differing primary dormancy states: primary dormant seeds and seeds that were cold stratified for 1, 2, 4 or 6 months.

One hundred seeds were mixed with saturated moist sand and placed in a paper box. The sand was kept fully saturated during cold stratification by adding distilled water to the sand every week. Several vent holes (1 cm diameter) were made in the wet sand containing seeds. The paper box was then placed in a growth chamber at 5 °C equipped with ventilation to ensure even air circulation. At each sampling time, three boxes (three replicates) were removed (12 boxes total for the four sampling times). The seeds in boxes were used to measure the levels of ABA, GA_{4+7} and GA_{1+3}. Distilled water was added as required during storage to ensure that moisture was a nonlimiting factor for primary dormancy release.

Preparation of seeds with different secondary dormancy states

Korean pine seeds enter complete secondary dormancy in late summer (August) and gradually release secondary dormancy in early autumn (September) and mid-autumn (October) after seed dispersal (Song et al. 2018). There were three secondary dormancy states for seeds: secondary dormant seeds (late summer after seed dispersal), seeds in early autumn after seed dispersal and seeds in mid-autumn after seed dispersal.

For obtaining seeds in these states, 14 nylon bags, each containing 70 freshly harvested seeds were buried at each of three locations in the *Pinus koraiensis* plantation forest in early November 2014. For preventing animals from eating the seeds, the nylon bags were placed into metal mesh before burial under the leaf litter on the soil surface. Two bags of seeds from each location were collected once a month from August to October 2015 and used to measure of the levels of AA, GA_{4+7} and GA_{1+3}.

Measurements of ABA, GA_{4+7} and GA_{1+3} during dormancy maintenance

The levels of ABA, GA_{4+7} and GA_{1+3} were determined in seeds with different primary dormancy states and seeds with different secondary dormancy states. The retrieved seeds were washed with distilled water and surface-dried with filter paper. Then, 20 seeds were placed in each of six replicate Petri dishes (9 cm) containing eight layers of filter paper pre-moistened with 12 ml of distilled water. The dishes were sealed with parafilm to reduce water loss. Distilled water was added as required. Plates were then placed in a growth chamber under germination conditions (25 °C, 14-h light/16 °C, 10-h dark). Seeds were sampled on day 0 after incubation (0DAI), 5 days after incubation (5DAI) and 11 days after incubation (11DAI) (when the radicle first protruded through the seed coat). Then, the levels of ABA, GA_{1+3}, and GA_{4+7} were measured in the embryo, megagametophyte and seed coat separately excised and immediately frozen, lyophilized, and stored at 20 °C until measurement using enzyme-linked immunosorbent assay (ELISA) (Yang et al. 2001). For ELISA, 0.2 g of the tissue sample was added to 6 ml of 80% methanol with 1 mmol L^{-1} butylated hydroxytoluene as an antioxidant, then homogenized and held at 4 °C for 4 h. The sample was then centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was passed through a C18 Sep-Pak cartridge (Waters Corp., Milford, MA, USA), then the eluate was dried in pure N_2 at 20 °C to remove the methanol. The N_2-dried extract was then dissolved in 2.0 ml of phosphate-buffered saline (containing 5 mL of 0.1% v/v Tween 20 and 0.5 g gelatin). Standards for GA and ABA, samples and antibodies were added to microtitration plates, and the plates were incubated at 37 °C for 30 min. Polyclonal antibodies against GA and ABA were generated in rabbits and purified as described by Weiler (1981). Horseradish peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from Sigma (St Louis, MO, USA) and used as a secondary antibody for each well, followed by incubation at 37 °C for 30 min. Next, enzyme–substrate *o*-phenylenediamine was purchased from Sigma (St Louis, MO, USA) and added to each well, and the plates were incubated in the dark at 37 °C for 15 min. Then, 2 mol L^{-1} H_2SO_4 was added to each well to terminate the reaction. The absorbance was recorded at 490 nm and used to calculate hormone concentrations using the methods of Weiler (1981).

Statistical analyses

One-way ANOVA and a least significant difference (LSD) test were used to determine differences among the levels of the respective hormones or hormone ratios (ABA, GA_{4+7}, and GA_{1+3} and the ratios of ABA/GA_{4+7} or ABA/GA_{1+3}) at

the three incubation times ($P < 0.05$). Changes in the ratios of ABA/GA_{4+7} and ABA/GA_{1+3} in the seed parts during dormancy (primary dormancy or secondary dormancy) release were also analyzed using a one-way ANOVA at $P = 0.05$. When data were not normally distributed and heterogeneity was observed after transformation, a nonparametric test (Mann–Whitney test) was performed using SPSS statistical software (version 19.0, IBM, Armonk, NY, USA).

Results

Changes in levels of ABA, GA_{4+7} and GA_{1+3} during incubation of seeds in different primary dormancy states

Compared with ABA levels at 0 DAI in embryo and megagametophyte in primary dormant seeds and seeds cold stratified for 6 months, levels did not change significantly at 11 DAI (Fig. 1a, b). However, ABA in embryos in seeds cold stratified for 4 months and in megagametophytes in seeds cold stratified for 2 months increased remarkably by 11 DAI (Fig. 1a, b). In contrast, ABA in seed coats at 11 DAI decreased in seeds in all five primary dormancy states (Fig. 1c).

For GA_{4+7}, levels in embryos were higher in primary dormant seeds and seeds cold stratified for 2 months, 4 months and 6 months at 11 DAI than at 0 DAI (Fig. 2a). Levels in megagametophytes and seed coats in seeds in the five primary dormancy states did not change significantly between 11 DAI and 0 DAI (Fig. 2b, c), except for a pronounced increase in megagametophytes in seeds cold stratified for 6 months (Fig. 2b).

For GA_{1+3}, levels in embryos, megagametophytes and seed coats in seeds in different primary dormancy states did not change significantly between 0 and 11 DAI (Fig. 3), with the exception of a significant increase in embryos in seeds cold stratified for 1 month (Fig. 3a), in megagametophytes in seeds cold stratified for 6 months (Fig. 3b), and a pronounced decrease in seed coats in seeds cold stratified for 2 months (Fig. 3c).

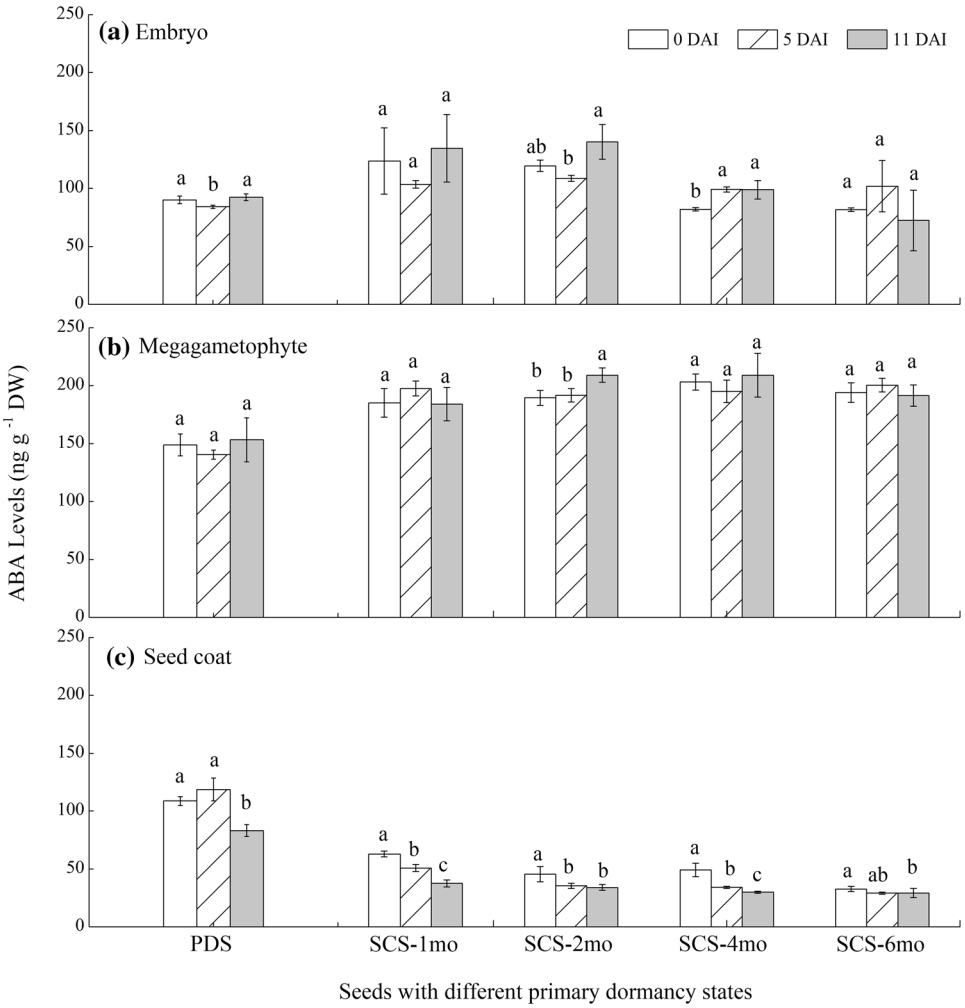

Fig. 1 Mean (±SD) ABA levels in seeds in different primary dormancy states during germination incubation. Different lower case letters indicate significant differences among incubation times. *PDS* primary dormant seeds, *SCS-1mo* seeds cold stratified for 1 month, *SCS-2mo* seeds cold stratified for 2 months, *SCS-4mo* seeds cold stratified for 4 months, and *SCS-6mo* seeds cold stratified for 6 months. *0DAI* 0 day after incubation, *5DAI* 5 days after incubation, and *11DAI* 11 days after incubation

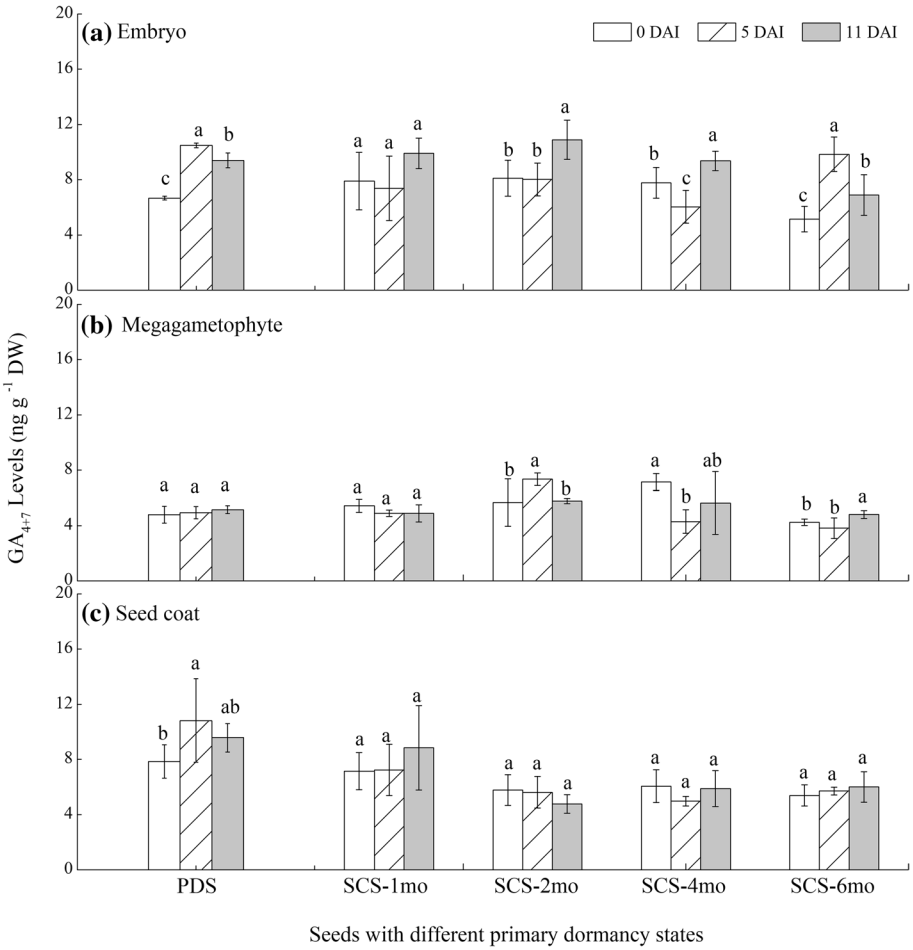

Fig. 2 Mean (±SD) GA_{4+7} levels of seeds with different primary dormancy states during germination incubation. Different lower case letters indicate significant differences among incubation times. The meanings of abbreviations are the same as that shown in Fig. 1

Changes in ABA, GA_{4+7} and GA_{1+3} during incubation of seeds in different secondary dormancy states

ABA levels by 11 DAI had significantly decreased in embryos of seeds in different secondary dormancy states (Fig. 4a), but megagametophyte levels did not change significantly (Fig. 4b). In seed coats, ABA differed significantly only in seeds in mid-autumn after dispersal; levels had decreased by 11 DAI (Fig. 4c).

GA_{4+7} levels in embryos and megagametophytes in secondary dormant seeds had significantly decreased by 35% and 20%, respectively, by 11 DAI (Fig. 4d, e). In megagametophytes and seed coats in seeds in early autumn after seed dispersal, levels had not changed significantly (Fig. 4e, f). In contrast, embryo and seed coat levels in seeds in mid-autumn after seed dispersal significantly increased by 134% and 17% (Fig. 4d,f), respectively, and megagametophyte levels also exhibited a slight increase ($P > 0.05$) (Fig. 4e).

GA_{1+3} in embryos and seed coats in secondary dormant seeds had significantly decreased by 24% and 18%, respectively, by 11 DAI (Fig. 4g, i). For seeds in early autumn after dispersal, levels in embryos did not change (Fig. 4g), but GA_{1+3} in megagametophytes gradually increased (Fig. 4h) significantly decreased in seed coats (Fig. 4i). GA_{1+3} also increased 38% by 5 DAI and 33% by 11 DAI in embryos in seeds in mid-autumn after dispersal (Fig. 4g), whereas levels in megagametophytes and seed coats did not differ significantly between 0 and 11 DAI (Fig. 4h, i).

Changes in ABA/GA_{4+7} and ABA/GA_{1+3} ratios during primary dormancy release and during incubation of seeds in different primary dormancy states

In seeds after 6 months of cold stratification, the ABA/GA_{4+7} ratio in embryos (Fig. 5a) and ABA/GA_{1+3} ratio in embryos and megagametophytes (Fig. 5d, e) did not differ significantly compared with those in primary dormant seeds ($P > 0.05$), but the ABA/GA_{4+7} ratio in megagametophytes (Fig. 5b) increased 46% ($P < 0.05$). ABA/GA_{4+7} and ABA/GA_{1+3} in seed coats significantly ($P < 0.05$) decreased as the cold stratification times increased (Fig. 5c, f).

After primary dormant seeds were transferred to germination conditions, by 11 DAI, the ratios of ABA/GA_{4+7} in

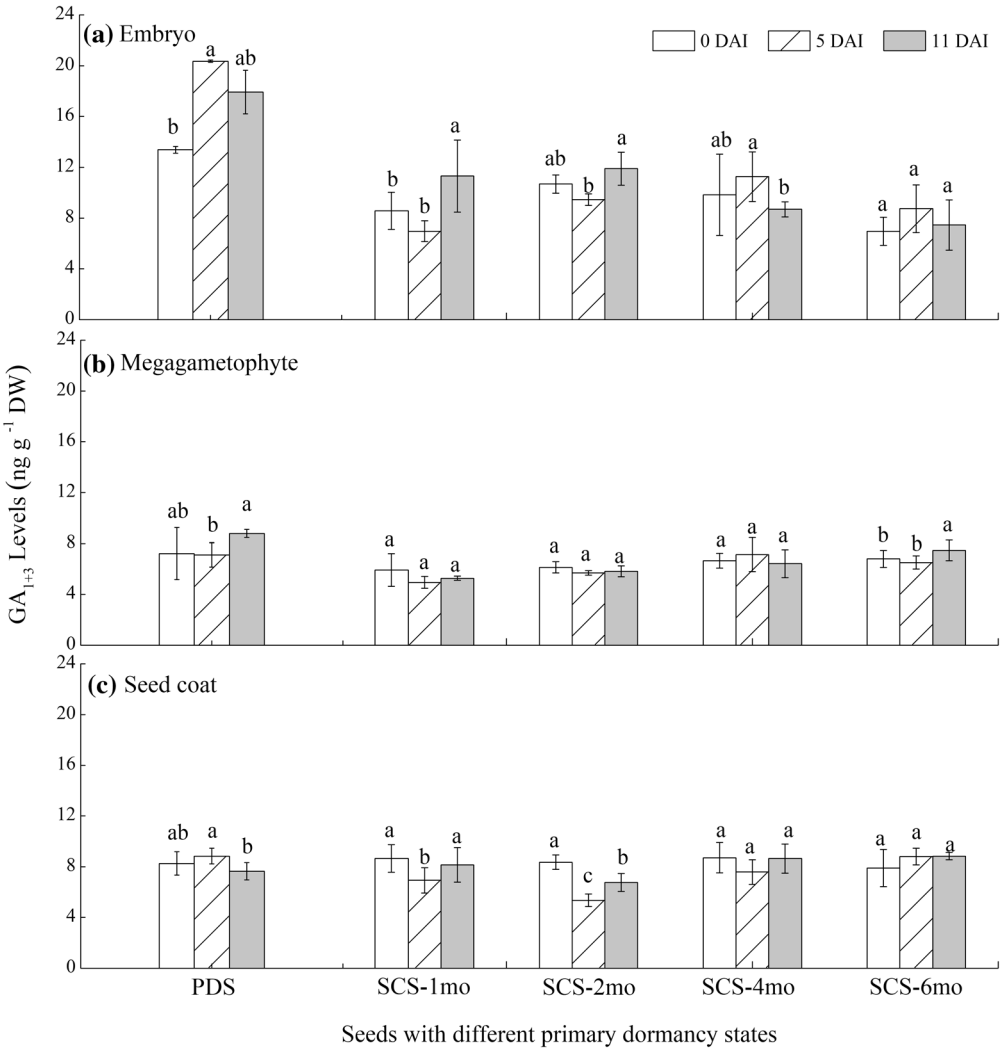

Fig. 3 Mean (±SD) GA_{1+3} levels of seeds with different primary dormancy states during germination incubation. Different lower case letters indicate significant differences among incubation times. The meanings of abbreviations are the same as that shown in Fig. 1

megagametophytes (Fig. 5b) and ABA/GA_{1+3} in embryos and megagametophytes (Fig. 5d, e) did not vary significantly compared with levels at 0 DAI ($P > 0.05$), but ABA/GA_{4+7} in embryos had significantly decreased ($P < 0.05$) (Fig. 5a). With two exceptions, ABA/GA_{4+7} and ABA/GA_{1+3} in embryos and megagametophytes of seeds cold stratified for 1, 2 or 4 months did not differ between 0 and 11 DAI (Fig. 5a, b, d, e) ($P > 0.05$); ABA/GA_{1+3} in the embryo of seeds cold stratified for 1 month significantly decreases ($P < 0.05$; Fig. 5d), but increased significantly ($P < 0.05$) in the megagametophyte of seeds cold stratified for 2 months (Fig. 5e). In contrast, ABA/GA_{4+7} and ABA/GA_{1+3} in embryos and megagametophytes of seeds cold stratified for 6 months had decreased significantly ($P < 0.05$) by 11 DAI compared with 0 DAI (Fig. 5a, b, d, e). ABA/GA_{4+7} and ABA/GA_{1+3} in seed coats of seeds in primary dormancy seeds and in the different states of primary dormancy were significantly lower ($P < 0.05$) at 11 DAI than at 0 DAI (Fig. 5c, f).

Changes in ABA/GA_{4+7} and ABA/GA_{1+3} during secondary dormancy release and during incubation of seeds in different secondary dormancy states

During the release of secondary dormancy, ratios of ABA/GA_{4+7} and ABA/GA_{1+3} significantly decreased ($P < 0.05$) in various parts of the seeds (Fig. 6). The ABA/GA_{4+7} ratio in embryos was a remarkable exception; it was 31% higher in seeds in mid-autumn after dispersal than in secondary dormant seeds (Fig. 6a).

The ratios of ABA/GA_{4+7} and ABA/GA_{1+3} in various parts of secondary dormant seeds increased during incubation (Fig. 6), except for a slight decrease in ABA/GA_{4+7} in seed coats (Fig. 6c). Specifically, ABA/GA_{4+7} increased significantly ($P < 0.05$) in embryos and megagametophytes (Fig. 6a, b). In contrast, by 11 DAI, in seeds with secondary

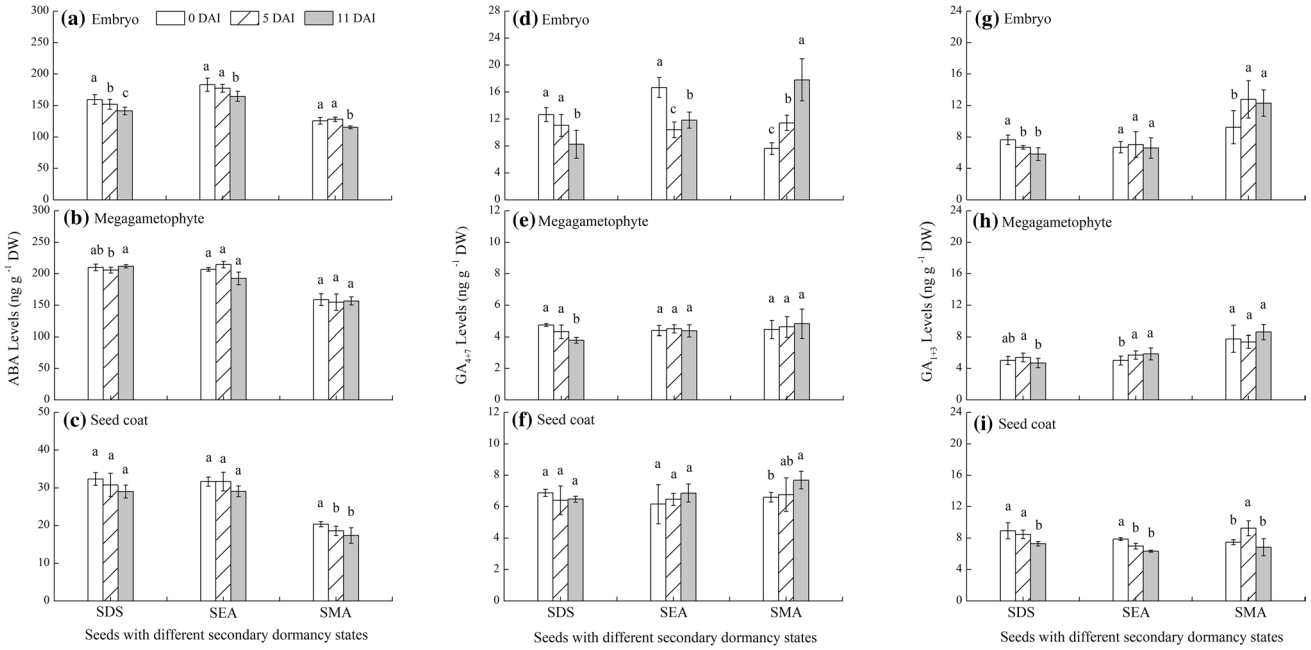

Fig. 4 Mean (±SD) ABA, GA_{4+7} and GA_{1+3} levels of seeds with different secondary dormancy states during germination incubation. Different lower case letters indicate significant differences among incubation times. SEA: seeds in the first early autumn after seed dispersal and SMA: seeds in the first mid-autumn after seed dispersal. *0DAI* 0 day after incubation, *5DAI* 5 days after incubation, and *11DAI* 11 days after incubation

dormancy already released (seeds in mid-autumn after dispersal), ABA/GA_{4+7} in embryos and seed coats (Fig. 6a, c) and the ratios of ABA/GA_{1+3} in the embryo and megagametophyte (Fig. 6d, e) had significantly decreased ($P < 0.05$).

Discussion

Dormancy is directly correlated with high endogenous ABA content in tree seeds (Feurtado et al. 2004; Chen et al. 2007). Cold stratification affects metabolic and physiological changes in seeds, including a rapid decline in ABA content and increase in GA, which are responsible for the reduced seed dormancy (Si et al. 2016; Wang et al. 2016; Ma et al. 2018). However, in Korean pine seeds, ABA levels in the embryo and megagametophyte were not reduced, nor did GA_{4+7} and GA_{1+3} increase when primary dormancy was released after 6 months of cold stratification. Several studies also failed to establish an association between hormone levels during cold stratification or after-ripening and dormancy states in a variety of species (Chae et al. 2004). For example, during cold stratification, both ABA and GA_3 in a significantly decreased in *Cotinus coggygria* seeds (Deng et al. 2016). Moreover, the levels of ABA, GA_1, GA_3, GA_4, and GA_7 in the seed coat and embryo of *Myrica rubra* seeds are lower following 8 weeks of warm stratification and 12 weeks of cold stratification compared with levels in freshly harvested seeds (Chen et al. 2008).

Recently, several studies revealed that the control of seed dormancy may involve the ratio of ABA/GA rather than the individual amounts of ABA or GA (Bicalho et al. 2015; Liu and Zang 2016). Although GA concentrations do not differ during warm stratification in *Taxus yunnanensis* seeds, a large decrease in ABA content reduces the ABA/GA ratio, which allows germination (Bian et al. 2018). Wang et al. (2018) also found that a decrease in the ABA/GA_3 ratio during 2 months of cold stratification correlates with the dormancy break of *Idesia polycarpa* seeds, although the levels of ABA and GA_3 are not significantly altered between 0 day and 2 months of cold stratification. However, the ABA/GA ratio in the embryo and megagametophyte of Korean pine seeds did not differ significantly during cold stratification of primary dormant seeds. However, the ratios of ABA/GA_{4+7} and ABA/GA_{1+3} in the seed coat significantly decreased during cold stratification. Seeds in which primary dormancy was already released had no significant increase in seed coat GA levels, but seed coat ABA concentrations were significantly lower compared to primary dormant seeds, resulting in a decreased AB/GA ratio in the seed coat. ABA leaching from the seed coat into the incubation medium appears to be the most important cause for the decline of seed coat ABA levels. However, this cause and effect needs to be further verified. In addition, the ABA/GA_{4+7} ratio in the megagametophyte and seed coat and the ABA/GA_{1+3} ratio in various parts of the seeds significantly decreased during the release

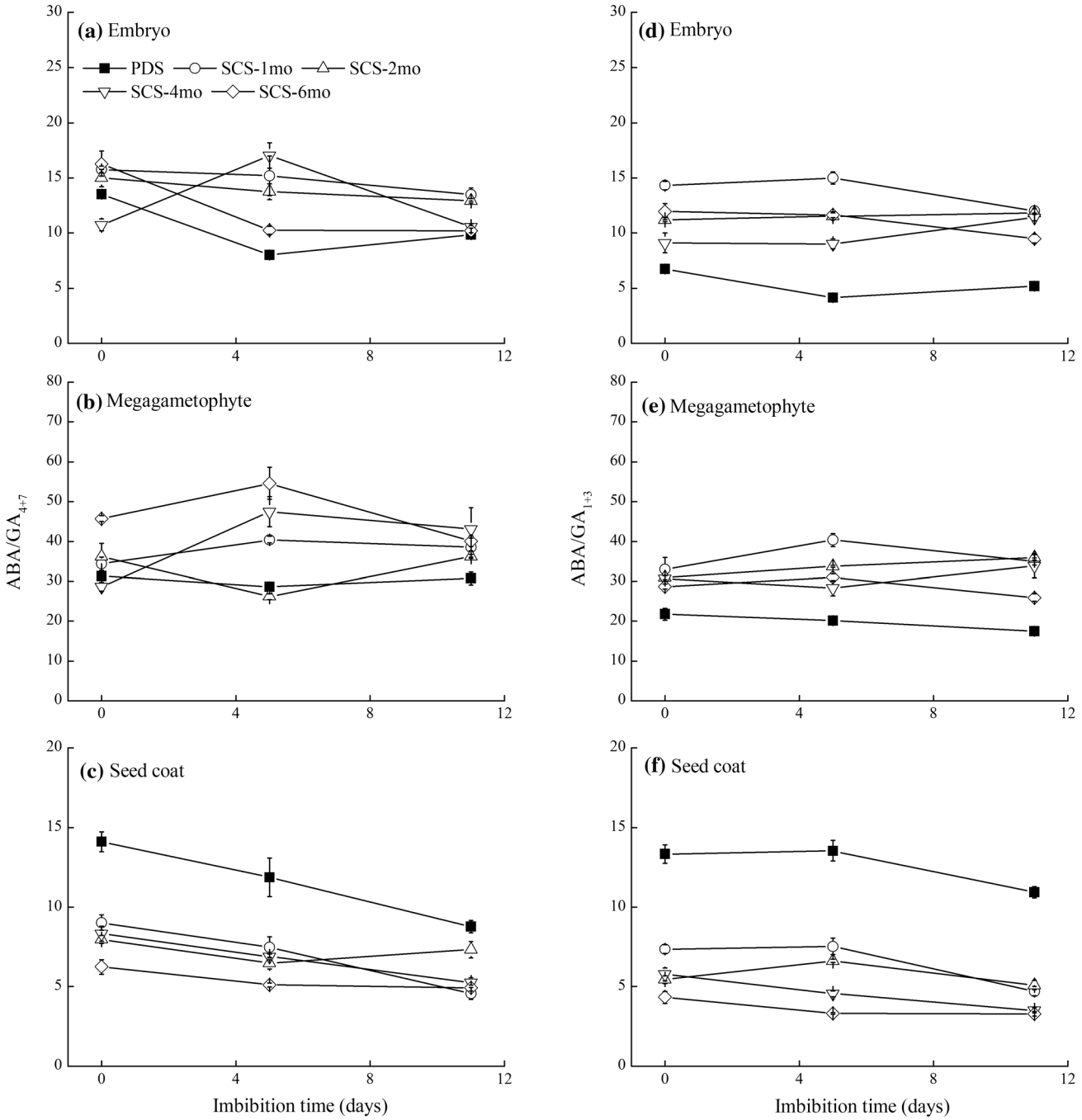

Fig. 5 Mean (±1 SE) ratios of ABA/GA_{4+7} in embryo (**a**), megagametophyte (**b**) and seed coat (**c**) of seeds with different primary dormancy states during germination incubation. Mean (±1 SE) ratios of ABA/GA_{1+3} in embryo (**d**), megagametophyte (**e**) and seed coat (**f**) of seeds with different primary dormancy states during germination incubation. Each value is the mean of three replicates. The meanings of abbreviations are the same as that shown in Fig. 1

of secondary dormancy. These results indicate that ABA/GA controls the release of secondary dormancy but is not involved in the regulation of primary dormancy release in Korean pine seeds. The changes in GA_{4+7} and GA_{1+3} in the present study do not represent the change in total GA content, and hence in the ABA/GA ratio. Moreover, changes in GA and ABA sensitivity can be used to determine the stages of dormancy loss that cannot be discerned with hormones levels or the ABA/GA ratio (Tuttle et al. 2015). Rodríguez et al. (2018) reported that ABA levels remain unchanged during dry storage at 25 °C, but sensitivity to ABA is reduced. Although absolute ABA levels and GA_{34} levels

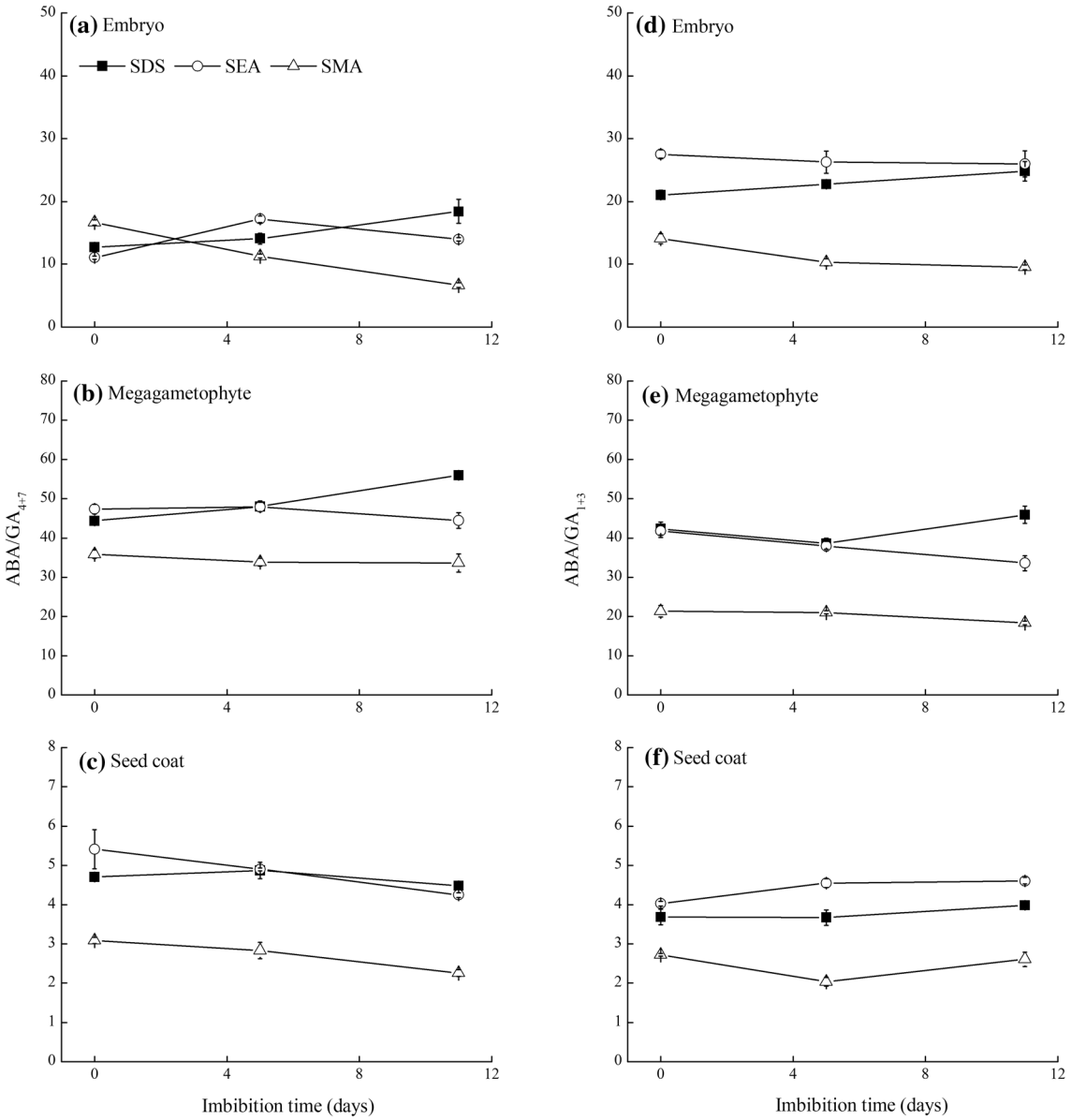

Fig. 6 Mean (±1 SE) ratios of ABA/GA_{4+7} embryo (**a**), megagametophyte (**b**) and seed coat (**c**) of seeds with different secondary dormancy states during germination incubation. Mean (±1 SE) ratios of ABA/GA_{1+3} in embryo (**d**), megagametophyte (**e**) and seed coat (**f**) of seeds with different secondary dormancy states during incubation. Each value is the mean of three replicates. The meanings of abbreviations are the same as that shown in Fig. 4

remain constant during cold stratification, the transcription of genes for ABA signaling components and sensitivity to ABA decline (Liu et al. 2015). Furthermore, Hauvermale et al. (2015) demonstrated that an increase in sensitivity to GA, but not in the biosynthesis of GA, during cold stratification breaks seed dormancy in GA-deficient mutants of *Arabidopsis thaliana*. Therefore, to accurately illustrate the regulatory mechanism underlying the release of seed primary dormancy of Korean pine seeds, we need to determine the sensitivity to ABA and GA during cold stratification.

Many studies have shown that ABA synthesis is necessary for dormancy maintenance (Bianco et al. 1997; Argyris et al. 2008; Ibarra et al. 2016). When dormant seeds and dormancy-released seeds are transferred to standard germination conditions, ABA levels drop rapidly in both, although to a lesser extent in dormant seeds than in nondormant seeds (Ali-Rachedi et al. 2004; Lee et al. 2010; Okamoto et al. 2010; Su et al. 2016). However, in dormant-imbibed seeds of *Arabidopsis* Cvi, Douglas-fir, and western white pine, the decrease in ABA is transient; ABA levels increase as dormancy maintenance continues (Bianco et al. 1997;

Corbineau et al. 2002; Ali-Rachedi et al. 2004; Feurtado et al. 2007). In contrast, during incubation in germination conditions, ABA levels greatly increase in nondormant seeds and cold-stratified seeds (Ali-Rachedi et al. 2004; Feurtado et al. 2004), and relatively lower levels of ABA are maintained in nondormant seeds (Feurtado et al. 2007). Hoang et al. (2013a) reported that embryo ABA levels decrease by approximately 4% during germination of nondormant seeds of *Hordeum vulgare*, whereas embryo ABA levels decrease more slowly during secondary dormancy maintenance. Therefore, the relative rate of ABA catabolism exceeds ABA biosynthesis during early imbibition/dormancy maintenance in seeds of some species. Once this transitory decrease period is over, ABA homeostasis favors ABA biosynthesis, and ABA levels increase during dormancy maintenance. This change in pattern of ABA levels appears to be a characteristic of early imbibition/dormancy maintenance in seeds of some species (Feurtado et al. 2007). However, our results revealed that the ABA levels in imbibed primary dormant seeds and in secondary dormant seeds do not have the transitory decrease and substantial later increase during 11 days of incubation in germination conditions, but perhaps our evaluations did not last long enough to detect such a pattern. ABA concentrations need to be determined in the remaining nongerminated seeds during the germination incubation. In addition, the ABA sensitivity of imbibed dormant seeds also regulates the maintenance of seed dormancy (Gubler et al. 2005; Tuttle et al. 2015). Although the ABA levels in primary dormant seeds and secondary dormant seeds did not increase substantially during incubation, the various parts of secondary dormant seeds and the seed coat of primary dormant seeds still maintained high ABA levels in Korean pine seeds. Thus, high seed coat ABA levels in primary dormant seeds and high ABA levels in secondary dormant seeds maintain primary and secondary dormancy, respectively.

The levels of GA_{4+7} and GA_{1+3} in embryos of primary dormant seeds significantly increased during incubation. Shu et al. (2013) also revealed that the expression of GA biosynthesis genes increases upon imbibition of primary dormant seeds of *Arabidopsis* ecotype Columbia-0. GA induces genes encoding enzymes involving in cell wall relaxation, and ABA acts as an antagonist (Bewley et al. 2013; Marowa et al. 2016). Reductions in ABA and increases in GA content are related to cell elongation (Dias et al. 2017). Therefore, it can be inferred that the rehydration of dry, primary dormant seeds during the germination incubation may lead to a decline in ABA and increase in GA content. After primary dormant seeds were cold stratified for 6 months, although the levels of ABA, GA_{4+7} and GA_{1+3} in various parts of seeds did not change greatly during incubation, the ratios of ABA/GA_{4+7} and ABA/GA_{1+3} significantly decreased. Thus, the ratio of ABA/GA, rather than the individual levels of the phytohormones, regulates germination of primary dormancy released seeds.

Although ABA levels did not increase during the germination incubation of secondary dormant seeds, the great decrease in GA_{4+7} and GA_{1+3} increased the ABA/GA ratio, which allowed the maintenance of secondary dormancy in Korean pine. In contrast, in seeds for which secondary dormancy was already released, the ABA levels decreased during incubation, and the GA_{4+7} and GA_{1+3} levels increased substantially, leading to a higher GA/ABA ratio. ABA, GA and GA/ABA coordinately regulate the germination of secondary dormancy released seeds. The maintenance of seed secondary dormancy in Korean pine involves the reduction of GA_{4+7}, GA_{1+3} and GA/ABA. This finding is consistent with the results of Hoang et al. (2013a), in which the gene expression involved in GA inactivation is reduced approximately twofold but remains high, while gene expression involved in GA synthesis is threefold lower during the maintenance of secondary dormancy in barley (*Hordeum vulgare* L., cv. Pewter). Ibarra et al. (2016) also showed that the induction of secondary dormancy of *Arabidopsis* seeds is related to changes in the GA content and sensitivity to GA. The results of many studies also documented that GA catabolism is important for entrance into secondary dormancy (Hoang et al. 2013a, b, 2014; Skubacz and Daszkowska-Golec 2017).

Although both primary dormant seeds and secondary dormant seeds have low germination percentages, the genes involved in ABA catabolism, GA biosynthesis, GA catabolism and ABA biosynthesis are different (Footitt et al. 2014). The influences of ABA and GA on the regulation of dormancy release and maintenance depend on the dormancy types of Korean pine seeds. Therefore, future work on seed dormancy mechanisms should focus on the individual seed dormancy types.

Conclusions

The regulatory mechanisms for ABA and GA during the maintenance of primary dormancy and secondary dormancy of Korean pine seeds are quite different. High levels of ABA in the seed coat maintain primary dormancy in Korean pine seeds, whereas GA_{4+7}, GA_{1+3} and the ABA/GA ratio are not important. In contrast, the maintenance of secondary dormancy in Korean pine seeds involves a reduction in GA_{4+7}, GA_{1+3} and GA/ABA and the retention of high ABA levels. Therefore, we should focus on the roles of ABA and GA in the separate seed dormancy type to fully understand the regulation of seed dormancy and improve regeneration during restoration of Korean pine forests.

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