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**Seed Dormancy and Germination of *Jeffersonia dubia* in  
Relation to Temperature, Hormone Levels, and Cell  
Wall Polysaccharides**

**Yong Ha Rhie**

*Department of Horticultural Science and Biotechnology,  
Seoul National University*

**ABSTRACT**

Germination ecophysiology and assay of cell wall sugar content and endo- $\beta$ -mannanase were conducted in seeds of *Jeffersonia dubia*. We also analyzed the levels of ABA during dormancy breaking and hormone interactions in relation to embryo growth and germination. The ripe seed of *J. dubia* contained an underdeveloped embryo and was permeable to water. In nature, seeds were dispersed in May, and while embryos began to grow in September and were fully elongated by late November. Germination started in March the next year and seedlings emerged soon after germination. In the laboratory experiments, incubation at high temperatures (constant 25°C; 25/15°C day/night) for at least 8 weeks was required to initiate embryo growth, while a transfer to moderate temperatures (20/10°C; 15/6°C) was needed for the completion of embryo growth. Minimum 8 weeks at 5°C was effective in overcoming the physiological dormancy and seed germination after the embryos were fully elongated. Gibberellic acid (GA<sub>3</sub>) treatment could substitute for the high temperature requirement, but not for the low temperature requirement. Based on the dormancy-

breaking requirements, it is confirmed that the seeds have deep simple morphophysiological dormancy (MPD). Although seeds require 10–11 months from seed dispersal to germination in nature, under controlled conditions they required only 3 months by treatment with  $1,000 \text{ mg}\cdot\text{L}^{-1}$  GA<sub>3</sub> followed by incubation at 15/6°C. This represents practical knowledge for the propagation of plants from seeds.

Chemical hydrolysis of endosperm cell walls of seeds showed that they were mainly composed of mannose, and smaller quantities of glucose and galactose. The endo-β-mannanase was not detected for the first three months when underdeveloped embryos hardly grew at all. The embryo started to grow after September and the activity of endo-β-mannanase increased in micropylar endosperm regions of the seeds. The erosion of the endosperm cell wall was observed at the lateral side around the embryo, while the micropylar endosperm showed to be no obvious signs of being collapsed or damaged. The increase of enzyme activity coincided with the increase in length of the embryo under the moderate temperature condition. The embryos stopped growing during the winter season for about 2-3 months and enzyme activity was also in a low state. The enzyme activity resumed to increase again when during germination occurred in early March. Then the micropylar endosperm rupture occurred and germination was completed. The seasonal pattern of endo-β-mannanase activity was unique in seeds with a deep simple morphophysiological dormancy (MPD) and different from that of physiological dormancy (PD), morphological dormancy (MD), or non-deep MPD.

Exogenously-applied GA or fluridone, an ABA biosynthesis inhibitor, promoted

embryo growth in seeds of *J. dubia*, but exogenously-applied ABA restrained the growth of embryos. Embryos of the seeds exhumed in November were fully grown, but dormant. Although these seeds required cold stratification, exogenously-applied GA or fluridone could substitute cold stratification and promoted the radicle protrusion. However, treatments with either ABA or paclobutrazol, a GA biosynthesis inhibitor, treatments suppressed seed germination even in those subjected to cold stratification for 8 weeks. The data support a GA and ABA balance mechanism that could be applied for germination of seeds with a deep simple MPD as well as a non-deep PD or non-dormancy.

Keywords: Berberidaceae, deep simple morphophysiological dormancy, endo- $\beta$ -mannanase, hormone balance theory, warm plus cold stratification

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## GENERAL INTRODUCTION

*Jeffersonia dubia* (Maxim.) Benth. & Hook. F. ex Baker & S. Moore (Berberidaceae) is a herbaceous perennial, distributed in northeastern Asia, ranging from southern Korea to northeast China and eastern Siberia (Hutchinson, 1920; Krestov et al., 2006). *J. dubia* is an early-flowering plant that develops flower shoots and leaves from the bulb at the base of plant. Reproductively mature individuals are 10–30 cm high, with light violet flowers and attractive heart-shaped leaves. Thus, this plant is regarded as one of the new ornamental crops for the garden (Huang, 1994). The *J. dubia* has the potential to be used for medicinal purposes, as well as ornamental planting. Extracts of *J. dubia* roots are used in folk medicine as antidotes for stomach aches (Bae, 2000). Duke and Ayensu (1985) purified a compound from *J. dubia* roots, named berberine, which was identified as a promising cholesterol-lowering drug that acts through pathways that are distinct from those of statins (Kong et al., 2004). However, in the Korean Peninsula, *J. dubia* is a very rare and threatened species with only a small population in deciduous woodlands due to the extensive collection and replacement of natural areas (Kim, 2006). Thus, it is important that efforts should be taken to prevent the disappearance of *J. dubia*. This plant can be reproduced through root cuttings, but this method is much more time-consuming, expensive for producing seedlings and reduces genetic variability of the future populations. Therefore, efficient seed-based propagation methods are needed. Seeds of *J. dubia* cannot immediately germinate after dispersal in late spring. It takes more than 9 months for seeds to germinate under field conditions. Moist chilling at 5°C for 8 weeks or gibberellic acid (GA) treatments could not increase the germination in seeds of *J. dubia* (Grushvitzky,

1967; Lee et al., 2007). More studies are necessary to determine the requirements for germination in seeds of *J. dubia*.

Seeds of many species in the temperate regions of the northern hemisphere have underdeveloped embryos that must grow inside the seed before germination occurs (Baskin and Baskin, 1998). If embryo growth and germination of these species are completed at suitable temperatures in about 30 days or less without a dormancy-breaking pretreatment, seeds will have morphological dormancy (MD) (Baskin and Baskin, 1988; Nikolaeva, 1977). However, if seeds with underdeveloped embryos require more than 30 days to complete germination, they have both morphological and physiological dormancy, which is known as morphophysiological dormancy (MPD) (Baskin and Baskin, 1998; Nikolaeva, 1977). An extensive study on the ecophysiology of dormancy and the germination of seeds of forest herbs with MPD has been conducted for species of the eastern deciduous forests of North America (Baskin and Baskin, 1988). Several studies have also been published on the requirements for breaking the dormancy of seeds of species with MPD in temperate deciduous forests in Europe and Japan (Kondo et al., 2011; Mondoni et al., 2012; Vandeloos and Van Assche, 2009). Many ornamental spring ephemerals native to Korea, including *J. dubia*, have seeds with MPD and cannot germinate for several months (Lee et al., 2012). However, few studies have been conducted on the phenology and temperature requirements for dormancy breaking and germination of seeds with underdeveloped embryos in species of the Korean Peninsula including *J. dubia*. This study was conducted to classify the seed dormancy of *J. dubia* and to promote the germination by temperatures and gibberellic acid (GA).

The comprehensive study on the changes in the endosperm weakening enzyme (endo- $\beta$ -mannanase) and plant hormones has been conducted for model species for seed dormancy research such as *Arabidopsis*, lettuce, tomato, tobacco, rice, and so on. However, these seeds have only a shallow dormancy and germination can be occurred within a short period (< 30 days). Moreover, some seeds exhibited a non-dormancy (e.g. afterripened) or conditional dormancy (Finch-Savage and Leubner-Metzger, 2006). At the level of endo- $\beta$ -mannanase and plant hormones during dormancy break little is known about MPD. MPD is thought to be the ancestral seed dormancy type in angiosperm and gymnosperm. Thus, the present study on the pattern of endo- $\beta$ -mannanase and plant hormones during dormancy breaking in seed of *J. dubia* was performed in an attempt to further understanding about the seed dormancy and germination in basal angiosperm.

# LITERATURE REVIEW

## Seed dormancy classification

The block of an intact viable seed to complete germination under favorable conditions is termed seed dormancy (Hilhorst, 1995; Bewley, 1997; Li and Foley, 1997). The timing of germination is species-specific and depends on several variables (e.g., the plant life cycle, habitat preference, and geographical distribution) (Nikolaeva, 1999). Nikolaeva (1977) described the dormancy types to create classification of the enormous variation in seed dormancy mechanisms. Based on the morphological and physiological characteristics of the seed, Baskin and Baskin (1998, 2004) devised a comprehensive classification system. This system includes five classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD). PD is the most common class of dormancy in gymnosperms and all major angiosperms, including *Arabidopsis thaliana*, lettuce, sunflower, tobacco, tomato, and several cereals (Baskin and Baskin, 1998). PD is caused by low growth potential of the embryo which cannot overcome mechanical constraint of seed, and divided into three levels of dormancy (non-deep, intermediate, and deep). Non-deep PD can be broken by GA treatment, after-ripening, and cold or warm stratification (Baskin and Baskin, 2004). However, deep PD cannot be broken by GA treatment, and several months of warm and/or cold stratification are required for germination. Seeds with MD contain the small (underdeveloped) embryo. These embryos are not dormant and just need time to grow until germination (< 30 days).

MPD is found in seeds with both MD and PD. MPD is found in primitive angiosperms and primitive gymnosperms, including the Apiaceae, Aquifoliaceae, Araceae, Berberidaceae, Liliaceae, Magnoliaceae, and Ranunculaceae (Grushvitzky, 1967; Baskin and Baskin, 1998). Some researchers have reported that the evolutionary trend of an increase in the relative embryo size of seeds has functional importance for the evolution of dormancy of angiosperms and gymnosperms (Baskin and Baskin, 1998, 2004; Nikolaeva, 2004). Baskin and Baskin (1998) suggested that the loss of PD in MPD seeds alters the dormancy class as MD, which upon gain in embryo size results in non-dormant seeds. MD or MPD are thought to be the ancestral dormancy type among seed plants and is the most primitive dormancy class (Finch-Savage and Leubner-Merzger, 2006).

### **Cell wall polysaccharides in seeds**

Plants accumulate the large amount of carbohydrate polymers, which favors their adaptation different environments. Starch is the most widespread, followed by fructans and cell wall polysaccharides (galctomannans, xyloglucans, and galactans). Whereas starch appears to have a storage function exclusively, fructans and cell wall polysaccharides seem to hold other functions. Fructans participate in the osmotic control of plant cells and tissues and the cell wall polysaccharides are linked to hardness (mannans in endosperms of seeds of palms, tomato and lettuce), water relationships (xyloglucan in cotyledons and galactomannan in endospermic legume seeds) and cell expansion (lupin galactans). The cell wall storage polysaccharides have been usually classified into three groups: mannans, xyloglucans and galactans (Reid

and Edwards 1995). This classification is based essentially on structure, the mannans being also divided into pure mannans, glucomannans and galactomannans (Handford et al., 2003; Wang et al., 2006). The pure mannans are artificially defined as having 90% or more mannan with a linear chain of  $\beta$ -(1 $\rightarrow$ 4)-linked manopyranosyl residues with up to 10% of the mannose residues substituted by single units of  $\alpha$ -(1 $\rightarrow$ 6)-linked galactoses. Mannans are found in monocotyledons (e.g. *Phoenix dactylifera* and *Phytelephas macrocarpa* (ivory nut mannan)) and in dicotyledons (Reid, 1985). Galactomannans are composed of a linear backbone of  $\beta$ -(1 $\rightarrow$ 4)-linked D-mannose residues to which D-galactose residues are attached by  $\alpha$ -(1 $\rightarrow$ 6)-linkages. The ratio mannose:galactose and the statistical distribution of galactosyl residues along the mannan backbone vary from species to species, being of chemotaxonomical value (Buckeridge and Dietrich, 1990). The three subfamilies of the Leguminosae (Caesalpinioideae, Mimosoideae and Faboideae) can be distinguished by the mannose:galactose ratios of their seed galactomannans.

### **Hormonal regulation of germination**

Several plant hormones are involved in the control of seed dormancy and germination (Kucera et al., 2005). Abscisic acid (ABA) and gibberellic acid (GA) are such hormones that play a prominent role in dormancy and germination control. ABA is a sesquiterpene hormone that maintains seed dormancy and inhibits seed germination, while GA is a diterpene hormone that releases seed dormancy and induces seed germination (Hilhorst and Karssen, 1992). There are considerable evidences that ABA is an important positive regulator of both the induction of dormancy and the

maintenance of the dormant state in imbibed seed. Afterripening, i.e. a period of dry storage at room temperature of freshly harvested, mature seeds, is a common method to facilitate dormancy release (Bewley, 1997). A decline in ABA content, decreased sensitivity to ABA and increased sensitivity to gibberellins (GA) are associated with the after-ripening-mediated transition from the dormant to the non-dormant state of many species (e.g. Hilhorst, 1995; Benech-Arnold et al., 1999; Beaudoin et al., 2000; Romagosa et al., 2001; Koornneef et al., 2002).

High ABA contents are present in the strongly dormant seeds of an *Arabidopsis* ecotype (Cape Verde Island, Cvi), but not in afterripened, non-dormant ones (Ali-Rachedi et al., 2004). A genome-wide analysis of the *Arabidopsis* transcriptome concluded that a delicate interplay between GA and ABA biosynthesis and catabolism determines whether the GA-ABA balance would favor dormancy or germination (Cadman et al., 2006). The key genes in this balance were suggested to be the *NCED* (9-*cis*-epoxycarotenoid dioxygenase) and *CYP707A* (ABA 8-hydroxylase) gene families for ABA synthesis and catabolism, respectively, and *GA3ox1* (GA 3-oxidase) and *GA2ox2* (GA 2-oxidase) for GA synthesis and degradation, respectively.

The carotenoid biosynthesis inhibitors, norflurazon and fluridone, have been used in experiments to inhibit ABA biosynthesis (e.g. Grappin et al., 2000; Ali-Rachedi et al., 2004; Chae et al., 2004; da Silva et al., 2004). Grappin et al. (2000) demonstrated that freshly harvested *Nicotiana plumbaginifolia* seeds have higher ABA content and sensitivity. Moreover, de novo ABA biosynthesis occurs in imbibed fresh, but not imbibed after-ripened seeds. Grappin et al. (2000) also added gibberellins (GA) or fluridone to the seeds, both of which prevented ABA biosynthesis and accelerated the

rate of germination. Simultaneous addition of GA + ABA or fluridone + ABA negated the germination-promoting effects. Expression studies of ABA-regulated genes in *N. tabacum* seeds are in agreement with decreased ABA content and sensitivity in after-ripened seeds, and suggest a common role for ABA during the afterripening-mediated release from dormancy and control of germination rate of *Nicotiana* species (Bove et al., 2005; Kucera et al., 2005). ABA inhibits embryo growth potential and endosperm cap weakening during coffee seed germination (da Silva et al., 2004). A transient rise in ABA content in the embryo was evident early during imbibition. ABA treatment inhibits, and fluridone treatment accelerates, radicle protrusion of coffee seeds.

Work with the strongly dormant *A. thaliana* ecotype Cvi shows that dormancy may depend on an intrinsic balance of GA and ABA biosynthesis and catabolism, which will determine the dominance of either of the hormones (Ali-Rachedi et al., 2004; Cadman et al., 2006). While PD release of Cvi seeds occurs effectively by after-ripening, stratification or inhibition of ABA biosynthesis, the addition of GA appears less effective. GA treatment of dormant Cvi seeds caused a transient increase in ABA concentration (Ali-Rachedi et al., 2004), suggesting that in dormant seeds a feedback mechanism exists that maintains a high ABA:GA ratio. Inhibition of GA biosynthesis during seed development mimics the effects of exogenous ABA, for example in suppressing vivipary. It appears to be the ABA:GA ratio, and not the absolute hormone contents, that controls germination. Thus, it seems that GA directly antagonizes ABA signalling during dormancy induction of cereal grains. Experiments with other species are needed to determine whether this is a general phenomenon (Finch-Savage and Leubner-Merzger, 2006).

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## CHAPTER I

# Seed Dormancy and Germination in *Jeffersonia dubia* (Berberidaceae) as Affected by Temperature and Gibberellic Acid

### ABSTRACT

The genus *Jeffersonia*, which contains only two species, has a trans-Atlantic disjunct distribution. The aims of this study were to determine the requirements for breaking dormancy and germination of *J. dubia* seeds and to compare its dormancy characteristics with those of the congener in eastern North America. Ripe seeds of *J. dubia* contained an underdeveloped embryo and were permeable to water. In nature, seeds were dispersed in May, while embryos began to grow in September and were fully elongated by late November. Germination started in March next year and seeds emerged as seedlings soon after germination. In the laboratory experiments, incubation at high temperatures (25°C; 25/15°C) for at least 8 weeks was required to initiate embryo growth, while a transfer to moderate temperatures (20/10°C; 15/6°C) was needed for the completion of embryo growth. At least 8 weeks at 5°C was effective in overcoming the physiological dormancy and germination in seeds after the embryos were fully elongated. Thus, both high and low temperatures were essential to break dormancy. Gibberellic acid (GA<sub>3</sub>) treatment could substitute for the high temperature

requirement, but not for the low temperature requirement. Based on the dormancy-breaking requirements, it is confirmed that the seeds have deep simple morphophysiological dormancy. This dormancy type is similar to that of seeds of the eastern North American species *J. diphylla*. Although seeds require 10–11 months from seed dispersal to germination in nature, under controlled conditions they required only 3 months after treatment with 1000 mg·L<sup>-1</sup> GA<sub>3</sub> followed by incubation at 15/6°C. This represents practical knowledge for the propagation of plants from seeds.

Keywords: Berberidaceae, deep simple morphophysiological dormancy, embryo growth, underdeveloped embryo, warm plus cold stratification

## INTRODUCTION

Seeds of many species in the temperate regions of the northern hemisphere have underdeveloped embryos that must grow inside the seed before germination occurs (Baskin and Baskin, 1998). If embryo growth and germination of these species are completed at suitable temperatures in about 30 days or less without a dormancy-breaking pretreatment, seeds will have morphological dormancy (MD) (Baskin and Baskin, 1988; Nikolaeva, 1977). However, if seeds with underdeveloped embryos require more than 30 days to complete germination, they have both morphological and physiological dormancy, which is known as morphophysiological dormancy (MPD) (Baskin and Baskin, 1998; Nikolaeva, 1977). An extensive study on the ecophysiology of dormancy and the germination of seeds of forest herbs with MPD has been conducted for species of the eastern deciduous forests of North America (Baskin and Baskin, 1988). Several studies have also been published on the requirements for breaking the dormancy of seeds of species with MPD in temperate deciduous forests in Europe and Japan (Kondo et al., 2011; Mondoni et al., 2012; Vandeloos and Van Assche, 2009). However, few studies have been conducted on the phenology and temperature requirements for dormancy breaking and germination of seeds with underdeveloped embryos in species of broad-leaved, deciduous forests in Korea (Lee et al., 2012).

The *Jeffersonia dubia* seeds cannot immediately germinate after dispersal in late spring. It takes more than 10–11 months for seeds of *J. dubia* to germinate under field conditions. The *J. dubia* seeds have an underdeveloped embryo at seed maturity

(dispersal), and this embryo has to grow inside the seed before the seeds can germinate (Grushvitzky, 1967; Lee et al., 2012). A study on the embryo growth of seeds of *J. dubia* indicated that embryo growth occurs at 20 °C (Grushvitzky, 1967). Gibberellins (GAs) are plant hormones that regulate growth and influence various developmental processes, including stem elongation, germination, dormancy, flowering, sex expression, enzyme induction and leaf senescence (Baskin and Baskin, 1998). Embryos of GA-treated seeds of *J. dubia* were fully developed after 1 month at 20 °C, but only 4% of the seeds germinated (Grushvitzky, 1967). Warm and/or cold stratification can break dormancy in seeds of many species (Baskin and Baskin, 1998). Periods of (moist) cold stratification (4–5 °C) for up to 30 days could increase germination percentage and rate in the herbaceous perennial species *Liriope spicata* (Lee et al., 2006) and *Megaleranthis saniculifolia* (Lee et al., 2003). However, moist chilling at 5 °C for 8 weeks could not increase the germination percentage in *J. dubia* (Lee et al., 2007). They did not explain that seeds of *J. dubia* require warm and/or cold stratification for germination. The genus *Jeffersonia* includes only two species that exhibit an eastern North American-eastern Asian disjunct distribution (Li, 1952). *J. diphylla* (L.) Pers. is native in northern temperate regions of North America, and the conditions required for breaking its dormancy and for germination have been well studied. Seeds of *J. diphylla* require warm stratification followed by cold stratification for dormancy breaking, their embryos grow at warm temperatures, and GA<sub>3</sub> substitutes for warm stratification (Baskin and Baskin, 1989).

Although there is some information on the embryo growth and germination of *J. dubia* seeds, a more elaborate study is required to determine the factors controlling the

dormancy and germination in this species. The specific objectives of the present study were (1) to describe the timing of embryo growth, germination and seedling emergence under outdoor conditions, (2) to experimentally define the temperature requirements for embryo growth and germination and (3) to determine if GA<sub>3</sub> can replace the temperature requirement for embryo growth and germination in this *taxon*. An additional experiment was performed to test the ability of GA at various temperatures to overcome dormancy.

## MATERIALS AND METHODS

### Seeds

Seed capsules of *J. dubia*, consisting of 10–15 seeds, were harvested from a population of *J. dubia* in Yong-in (37°05' N, 127°24' E), Geonggi, Korea on 26 May 2009, 28 May 2010 and 22 May 2011. Here, *J. dubia* grows in a mesic deciduous woodland in Hantaek Botanical Garden that is an *ex situ* conservation habitat. Seeds were removed from the capsules by hand, winnowed and then air-dried at room temperature (22–25°C) for 2–4 days. Unless otherwise noted, experiments started within 1 week after harvest. Seeds not used immediately in the experiments were put into paper envelopes at 5°C until they were used in germination tests and for observations on embryo growth.

### Imbibition of seeds

This experiment was performed to determine if seeds have physical dormancy (PY). Three replications of 30 seeds collected in 2009 were placed on filter paper moistened with distilled water in 9-cm Petri dishes and kept in the laboratory at room temperature (22–25°C). Seeds were weighed to the nearest 0.1 mg at various times after incubation for 60 h. Seed mass was determined at 0.5-h intervals for the first 12 h, and then every 12 h for the next 60 h. Percent water uptake (% $W_s$ ) was calculated as % $W_s = [(W_i - W_d)/W_d] \times 100$ , where  $W_s$  = increase in mass of seeds,  $W_i$  = mass of seeds after a given interval of imbibition and  $W_d$  = mass of dry seeds.

### **Phenology of embryo growth, germination and seedling emergence**

The aim of these studies was to describe the phenology of embryo growth, germination and seedling emergence from seeds kept under near-natural temperature conditions. Seeds were placed in fine-mesh polyester bags and buried in pots or trays filled with potting soil on 8 June 2010. Pots and trays were placed in an experimental garden in the campus of Seoul National University, Seoul, Korea. The soil temperature at a depth of 3 cm was measured every 30 min with a thermo data logger (Watch Dog Model 450, Spectrum Technologies, Inc., Plainfield, IL, USA), and the weekly maximum and minimum temperatures were calculated (Fig. I-2).

Each of the 25 fine-mesh polyester bags filled with 20 seeds and 10 g of white sand was buried at a depth of 3 cm in the experimental garden. On the 8th and 23rd day of every month, ten seeds were removed randomly from one bag and the embryo length was measured from 8 June 2010 to 23 February 2011. Seeds were cut in half under a dissecting microscope by using a razor blade, and the embryo length was measured with an ocular micrometer. The ratio of embryo length to seed length (E:S ratio) was calculated.

Germination and seedling emergence phenology was monitored in seeds buried under natural conditions in June 2010. Each of the three fine-mesh polyester bags filled with 100 seeds was buried at a depth of 3 cm in the experimental garden. Seeds with an emerged radicle were counted and removed weekly for 1 year. A seed was considered germinated when its radicle protrusion occurs and reached 2 mm. Intact seeds that had not germinated were buried again.

Timing of seedling emergence was determined by sowing three replicates of 50

seeds 1 cm deep in plastic pots filled with potting soil. Three replicate pots were buried at soil level in a shady site in the garden. The experiment lasted for 1 year during which emerged seedlings were counted and removed weekly.

### **Laboratory experiments**

Seeds were treated with  $500 \text{ mg}\cdot\text{L}^{-1}$  of benomyl for 3 h for bacterial control before being used in laboratory experiments. Unless otherwise stated, each of the three replicates of 30 seeds each was placed in 9.0 cm diameter plastic Petri dishes lined with two layers of filter paper for temperature studies or with sand for GA studies. The sand used in these experiments was washed with distilled water and sterilized in hot air oven at  $80^{\circ}\text{C}$  for 48 h. Petri dishes were moistened with distilled water and sealed with parafilm (Pechiney Plastic Packaging, Menasha, WI, USA) to retard water loss during incubation. Experiments were conducted in light- and temperature-controlled incubators using a 12-h daily photoperiod (photosynthetically active radiation  $30\text{--}40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , with light provided by cool white fluorescent lamps). In the alternating regime, the high temperature was given for 12 h with light each day, and the low temperature for 12 h under darkness. At each observation, seeds with an emerged radicle were recorded and removed from the dishes; water was added as needed to keep the seeds moist. The experiments were terminated when no additional germination had occurred for 3 weeks.

### **Effect of temperature regimes on germination and embryo growth**

To determine whether seeds have MD or MPD, seeds were incubated at constant  $5^{\circ}\text{C}$ ,

and at alternating temperature regimes of 15/6, 20/10 and 25/15°C on 2 June 2010. Observations on germination were made at 7-day intervals. The experiment was terminated 60 weeks after seed incubation. In the experiments looking at embryo growth, ten seeds were removed at random from extra dishes for each temperature treatment at 4-week intervals for 52 weeks, and the E:S ratio of the seeds was measured as previously described.

### **Effect of warm-to-cold and cold-to-warm temperature sequences on germination and embryo growth**

The purpose of this experiment was to determine if warm, cold or warm plus cold stratification treatments were required for embryo growth and germination. On 2 June 2010, seeds were subjected to the following two temperature sequences: (1) beginning with warm temperature 25/15°C for 12 weeks → 20/10°C for 4 weeks → 15/6°C for 4 weeks → 5°C for 12 weeks, then moving the seeds back to 15/6, 20/10 and 25/15°C and continuing the sequence if they had not germinated; and (2) beginning with cold temperature 5°C for 12 weeks → 15/6°C for 4 weeks → 20/10°C for 4 weeks → 25/15°C for 12 weeks → 20/10°C for 4 weeks → 15/6°C for 4 weeks → 5°C for 12 weeks, then moving the seeds back to 15/6, 20/10 and 25/15°C and continuing the sequence if they had not germinated. E:S ratio and germination were measured as previously described.

### **High temperature requirements for germination**

This study determined the optimum temperature range and duration for warm

stratification. On 7 June 2011, seeds collected on May 2011 were firstly placed at 20/10, 25/15, 20 or 25°C for 2, 4, 6, 8 or 12 weeks. After each temperature × duration condition, all seeds were moved through the temperature sequence for germination. The sequence was 20/10°C for 4 weeks → 15/6°C for 4 weeks → 5°C for 12 weeks → 15/6°C for 4 weeks. This sequence of temperatures simulates natural conditions whereby high summer temperatures are followed by autumn temperatures and lower winter temperatures. Germination was measured at the end of incubation at 15/6°C.

### **Cold temperature requirements for germination**

The purpose of this study was to determine if cold stratification is required for radicle emergence in fully embryo-elongated seeds, and if so, how many weeks of cold stratification are required. On 2 June 2010, seeds were put in a fine-mesh polyester bag and placed in an experimental garden. On 10 November 2010, seeds were removed from their bag. The average E:S ratio of these seeds was  $0.675 \pm 0.012$  (mean  $\pm$  SE,  $n = 10$ ). These seeds were transferred to 5°C for 0, 2, 4, 6, 8, 10 or 12 weeks. Thereafter, seeds were incubated at 20/10°C and checked for germination after 42 days.

### **The role of GA<sub>3</sub> in breaking dormancy**

The ability of GA<sub>3</sub> to break dormancy is used to classify seed dormancy types (Baskin and Baskin, 1998). On 9 June 2010, seeds were soaked with distilled water (control) or in solutions of 10, 100 or 1000 mg·L<sup>-1</sup> GA<sub>3</sub> for 24 h at room temperature prior to incubation. All seeds were incubated at constant 20°C. After 0, 2, 4, 8 and 12 weeks, the E:S ratio of each seed was measured. At the end of the 12-week incubation

period, germination was measured.

### **Effect of GA<sub>3</sub> on germination under various incubation temperatures**

In this part of the study, we analysed the effect of GA<sub>3</sub> and incubation temperature on final germination of seeds. On 7 June 2011, seeds were soaked in solutions at concentrations of 10, 100 and 1000 mg·L<sup>-1</sup> GA<sub>3</sub>, or with distilled water (control) for 24 h at room temperature, and then incubated at 5, 15/6, 20/10 and 25/15°C for 12 weeks.

### **Statistical analysis**

The percent germination after pre-treatment at different temperatures was analyzed using ANOVA followed by Tukey's multiple comparison test (SAS Institute Inc., Cary, NC, USA). A two-way ANOVA was also used to test the interaction effect between GA<sub>3</sub> concentration and incubation temperature on percent seed germination.

## RESULTS

### Imbibition of seeds

Seeds of *J. dubia* imbibed water readily and followed a typical pattern of initial rapid water uptake with the seed mass increasing by  $113.6 \pm 4.0\%$  (mean  $\pm$  SE) after 6 h (Fig. I-1B), and  $144.1 \pm 6.4\%$  after 60 h; the seed mass stabilized beyond this point (Fig. I-1A).

### Phenology of embryo growth, germination and seedling emergence

The initial embryo length in June 2010 was  $0.40 \pm 0.02$  mm (mean  $\pm$  SE), in which the E:S ratio was 0.085 (Fig. I-2). Embryos hardly grew during the summer when temperatures were high. The E:S ratio on 8 September 2010 was 0.115. However, between 8 September and 23 November 2010, the E:S ratio increased rapidly to 0.930, in which the mean maximum and minimum soil temperatures during the preceding week were  $17.7^{\circ}\text{C}$  and  $14.1^{\circ}\text{C}$ , respectively. Embryos grew very little after 23 November 2010. Germination began between 23 February and 8 March 2011. On 8 March,  $12.5 \pm 2.3\%$  of the exhumed seeds had germinated (Fig. I-2). Three weeks later, on 27 March 2011,  $94.7 \pm 4.7\%$  of the seeds had already germinated. On 27 March 2011, emerged seedlings were first observed from  $14.8 \pm 6.4\%$  of the seeds, and the mean maximum and minimum temperatures during the preceding week were  $10.9^{\circ}\text{C}$  and  $4.7^{\circ}\text{C}$ , respectively. By the end of April 2011, seedlings had emerged from  $88.9 \pm 11.1\%$  of the seeds.

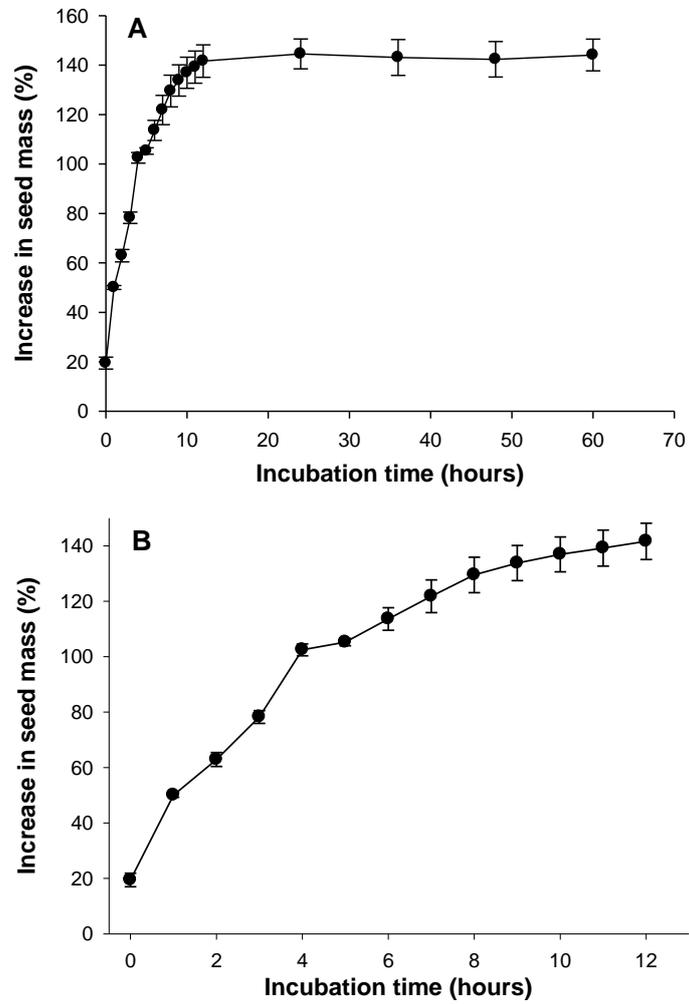


Fig. I-1. Water uptake by intact seeds of *Jeffersonia dubia* incubated at room temperature (22–25°C) on filter paper moistened with distilled water for 0–60 h (A) and for 0–12 h (B). Vertical bars represent SE.

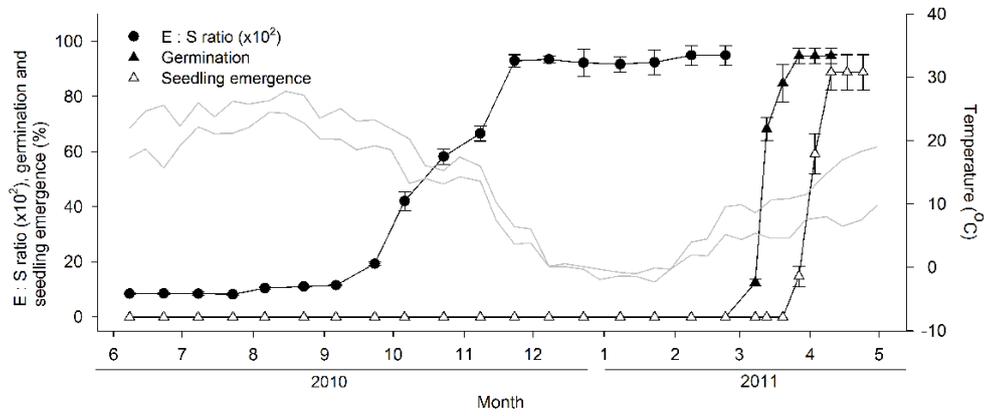


Fig. I-2. Embryo growth, mean percentage germination and seedling emergence of *Jeffersonia dubia* seeds sown at a depth of 3 cm. Dotted lines indicate mean weekly maximum and minimum soil temperatures at a depth of 3 cm. Vertical bars represent SE. The E:S ratio is the ratio of embryo length to seed length.

### **Effect of temperature regimes on germination and embryo growth**

No seeds germinated within 52 weeks at the constant temperature of 5°C, or at any of the three daily alternating temperature regimes of 15/6, 20/10 or 25/15°C (Fig. I-3). Even at 52 weeks after sowing, embryos did not grow at 5, 15/6 or 20/10°C. At 25/15°C, embryo growth began between weeks 8 and 12, by which time they had more than doubled in length. However, the E:S ratio increased to only 0.471 after 52 weeks.

### **Effect of warm-to-cold and cold-to-warm temperature sequences on germination and embryo growth**

For seeds incubated in the temperature sequence beginning with 25/15°C, the E:S ratio increased to 0.176 during the 12 weeks of incubation at 25/15°C (Fig. I-4A). A rapid increase in the embryo length was observed upon transfer from 25/15°C to 20/10 and 15/6°C. During 8 weeks of incubation at 20/10 and 15/6°C, the E:S ratio increased to 0.719. After 8 weeks at 5°C, the E:S ratio increased to 0.865. At this time, the first seed germinated. Germination began near the end of the 5°C treatment, and increased rapidly to  $84.9 \pm 3.63\%$  at 15/6°C, and then to  $88.0 \pm 4.44\%$  at 20/10°C. In the temperature sequence beginning at 5°C, embryos did not grow until they were moved to 25/15°C (Fig. I-4B). Embryo growth began after 8 weeks at 25/15°C and the E:S ratio increased to 0.517 at 20/10°C and then to 0.756 at 15/6°C. No seeds germinated after 48 weeks of incubation in the temperature sequence that began at 5°C. Seeds germinated to  $22.6 \pm 5.52\%$  during the second exposure to 5°C, reaching  $86.6 \pm 4.63\%$  shortly after the seeds were transferred to 15/6°C.

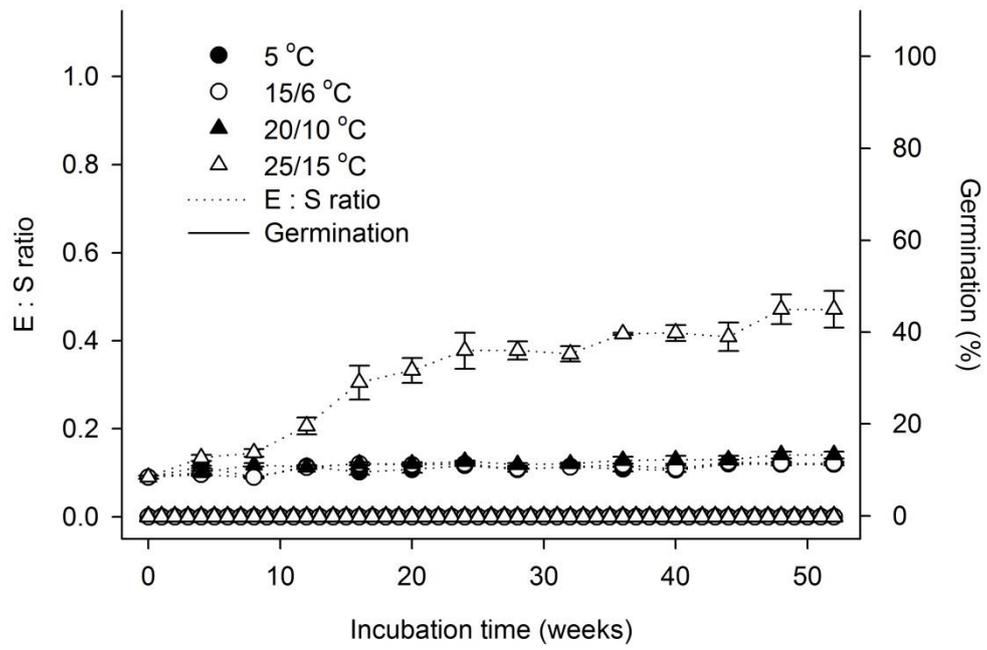


Fig. I-3. Embryo growth (dotted line) and germination (solid line) in seeds of *Jeffersonia dubia* incubated under various temperature regimes. Vertical bars represent SE.

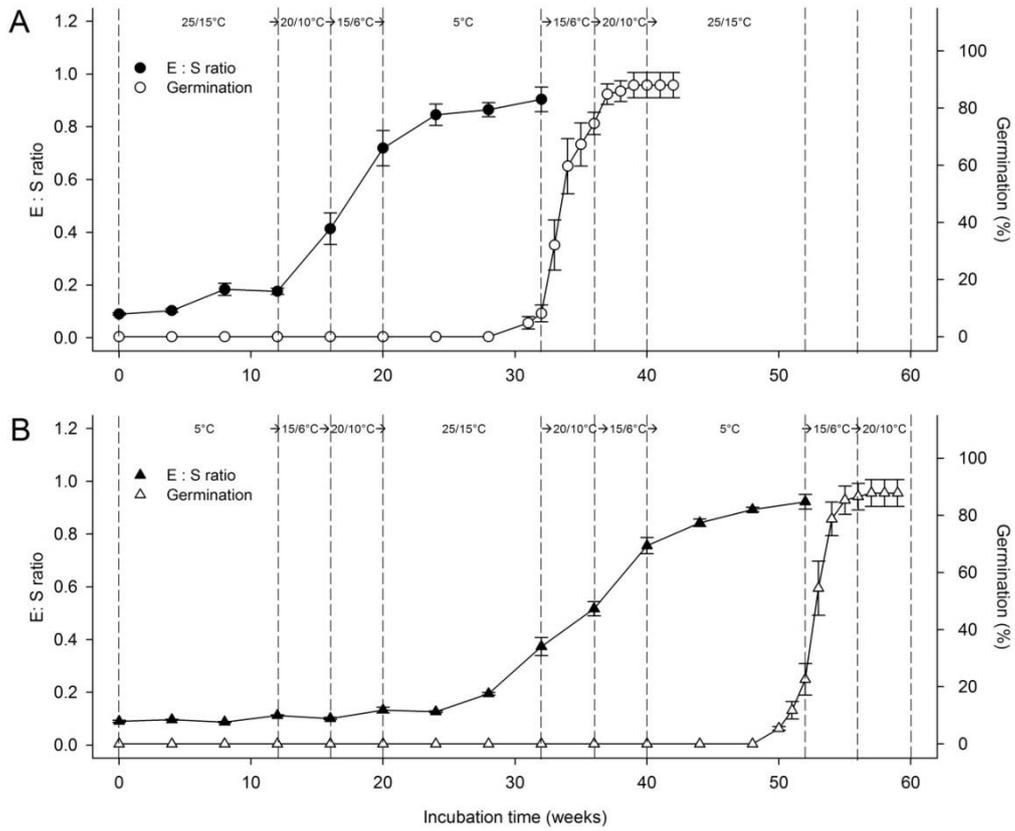


Fig. I-4. Embryo growth and germination in seeds of *Jeffersonia dubia* incubated under a temperature sequence beginning at 25/15°C (day/night) (A) or at constant 5°C (B). Vertical bars represent SE.

### **High temperature requirements for germination**

The temperature and duration of the pre-treatment significantly ( $P < 0.05$ ) affected the final germination percentage after the sequence of temperatures [20/10°C (4 weeks) → 15/6°C (4 weeks) → 5°C (12 weeks) → 15/6°C (4 weeks)] (Table I-1). More than 80% of the seeds germinated when seeds were warm stratified at 25/15 or 25°C for 8 weeks or at 20°C for 12 weeks. However, few seeds (< 11%) germinated after a pre-treatment at 20/10°C.

### **Cold temperature requirements for germination**

Seeds with fully elongated embryos required a prolonged exposure to cold stratification (5°C) to reach maximum germination (Fig. I-5). Germination was 0% for seeds cold stratified at 5°C for  $\leq 2$  weeks and then incubated at 20/10°C. However, seeds stratified at 5°C for 8, 10 or 12 weeks germinated to  $76.7 \pm 6.67$ ,  $83.2 \pm 8.15$  or  $95.0 \pm 5.00\%$  respectively.

### **The role of GA<sub>3</sub> in breaking dormancy**

The embryo length increased with an increase in GA<sub>3</sub> concentration (Fig. I-6A). However, when seeds were pre-treated with 0 (control), 10, 100 and 1000 mg·L<sup>-1</sup> GA<sub>3</sub> and re-incubated at 20°C for 20 weeks, 0, 0,  $3.2 \pm 1.01$  and  $10.7 \pm 5.0\%$  of the seeds germinated, respectively (Fig. I-6B).

Table I-1. Germination percentage of *Jeffersonia dubia* seeds after 4 weeks at 20/10°C (day/night) → 4 weeks at 15/6°C → 12 weeks at constant 5°C → 4 weeks at 15/6°C, following a pre-treatment for 0, 2, 4, 6, 8 or 12 weeks under four different temperature conditions.

Duration (weeks)	Pre-treatment temperature							
	20/10°C		20°C		25/15°C		25°C	
0	0.0 ± 0.0	a <sup>z</sup>	0.0 ± 0.0	a	0.0 ± 0.0	a	0.0 ± 0.0	a
2	0.0 ± 0.0	a	0.0 ± 0.0	a	0.0 ± 0.0	a	6.0 ± 0.7	a
4	0.0 ± 0.0	a	5.0 ± 5.0	a	25.7 ± 3.7	a	22.6 ± 6.8	a
6	0.0 ± 0.0	a	31.9 ± 6.9	ab	64.1 ± 5.4	b	49.5 ± 16.5	ab
8	6.9 ± 6.9	a	55.0 ± 10.0	bc	89.3 ± 10.3	b	88.4 ± 0.8	b
12	11.0 ± 11.0	a	82.0 ± 0.8	c	86.3 ± 6.3	b	92.5 ± 2.5	b

<sup>z</sup>An one-way Anova test was conducted in order to detect significant differences among weeks under the same temperature regime. Mean followed by the same letter within a column are not significantly different at  $P < 0.05$  by Tukey's multiple comparisons test.

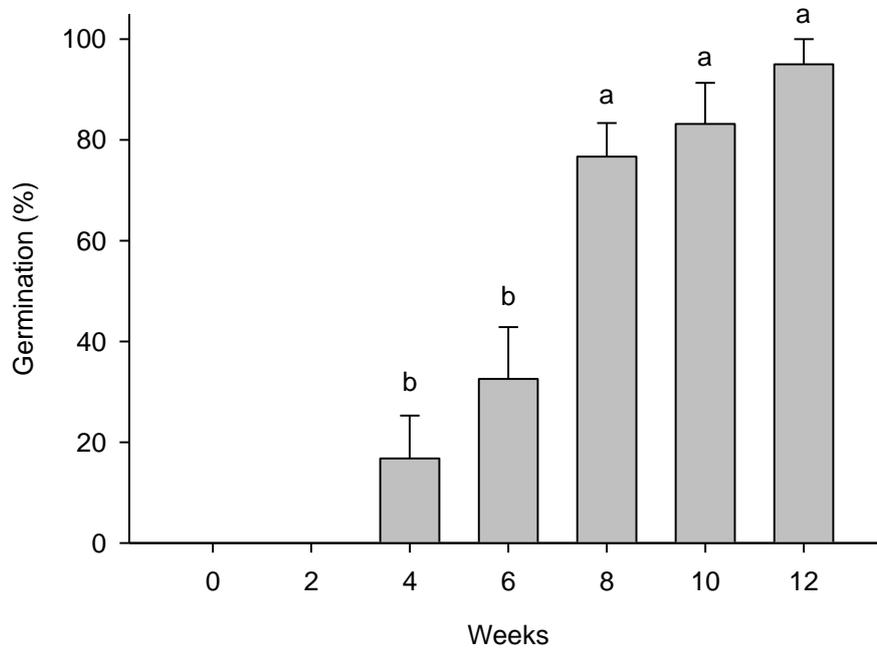


Fig. I-5. Germination percentage of *Jeffersonia dubia* seeds kept in fine-mesh polyester bags under field conditions until November 2010 and then placed at constant 5°C for 0, 2, 4, 6, 8, 10 or 12 weeks and then moved to 20/10°C (day/night) for 6 weeks. Vertical bars represent SEs. Bars with the same letter are not significantly different at  $P < 0.05$  by Tukey's multiple comparisons test.

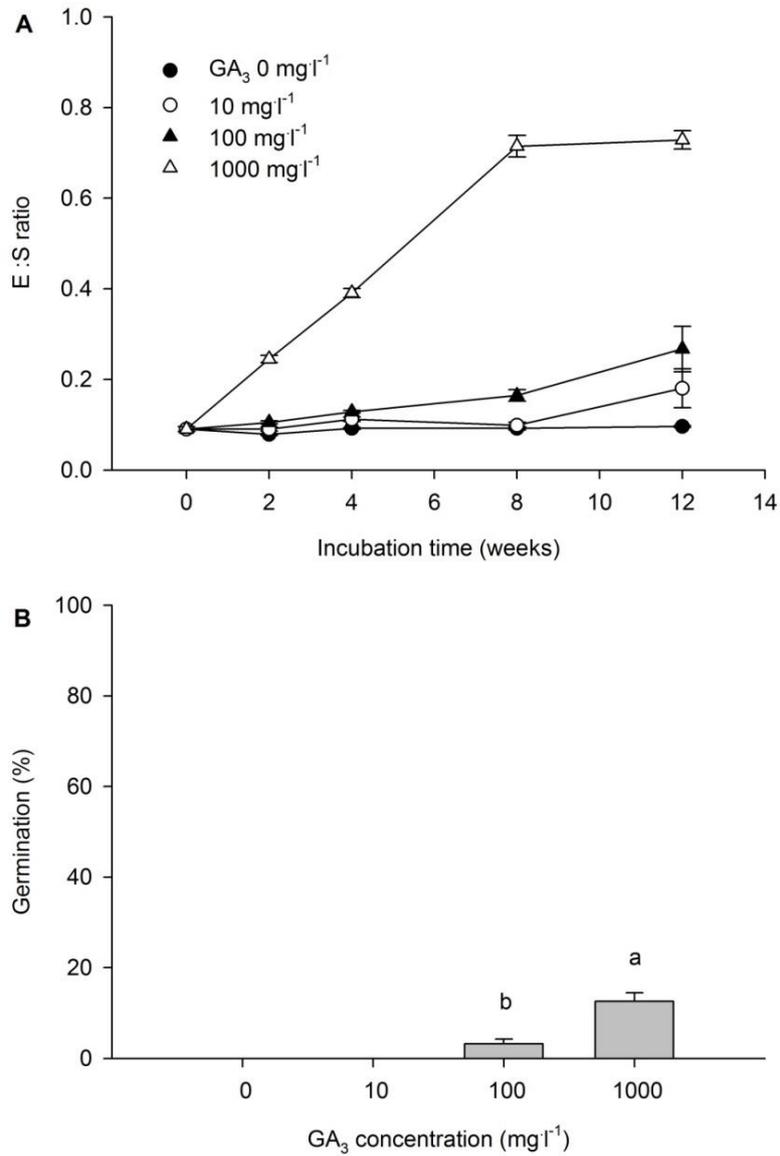


Fig. I-6. Embryo growth (A) and germination (B) of *Jeffersonia dubia* seeds at 20°C as affected by GA<sub>3</sub> concentrations. Treated seeds were soaked in a GA<sub>3</sub> solution for 24 h before incubation. Vertical bars represent SE. In (B) bars with the dissimilar letter are significantly different at  $P < 0.05$  by Tukey's multiple comparisons test.

### **Effect of GA<sub>3</sub> on germination under various incubation temperatures**

The concentration of GA<sub>3</sub> and the temperature conditions had significant effects on the final germination percentage after 12 weeks of incubation ( $P < 0.05$ ) (Fig. I-7). For all temperature conditions, the final germination percentage increased with increasing concentration of GA<sub>3</sub>. The germination percentage was highest for seeds incubated at 15/6°C, while seeds incubated at 25/15°C had very low germinating percentages. The final germination proportions recorded at 25/15, 20/10, 15/6 and 5°C were  $10.6 \pm 1.73$ ,  $30.1 \pm 5.86$ ,  $74.2 \pm 2.98$  and  $23.5 \pm 2.75\%$  in  $1,000 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub>, respectively.

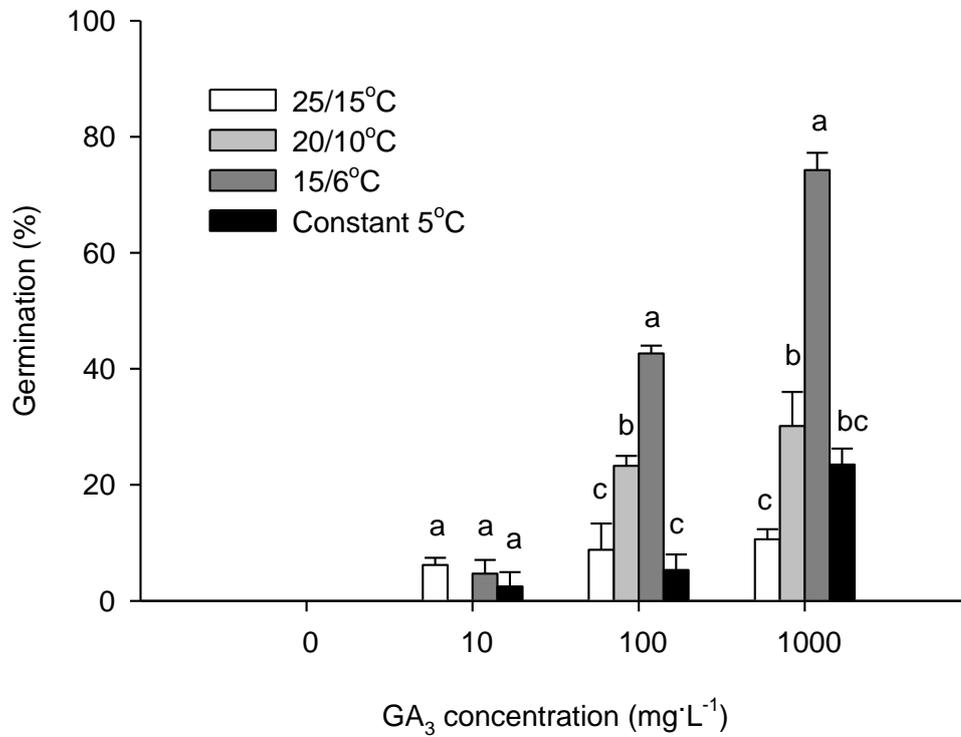


Fig. I-7. Percent germination of *Jeffersonia dubia* seeds after 12 weeks of incubation at 25/15 (day/night), 20/10, 15/6 and 5°C as affected by GA<sub>3</sub>. Treated seeds were soaked in a GA<sub>3</sub> solution for 24 h before incubation. Vertical bars represent SE. Bars with the same letter are not significantly different at  $P < 0.05$  by Tukey's multiple comparisons test.

## DISCUSSION

Seeds of *J. dubia* imbibed water readily, increasing in mass by 113.6% after 6 h under *ex situ* conditions, thus having no PY (Fig. I-1). Seeds have an underdeveloped embryo at the time of seed dispersal, thus they have morphological dormancy (MD) (Baskin and Baskin, 1998; Lee et al., 2012). According to Baskin and Baskin (1998), seeds that begin to germinate within 30 days only have MD, and embryos begin to grow soon after the seeds imbibe water at favorable temperatures. However, none of the freshly harvested seeds of *J. dubia* germinated after several months of incubation at the temperatures of 25/15, 20/10, 15/6 or 5°C, indicating that they have morphophysiological dormancy (MPD) as well as MD (Fig. I-3).

Baskin and Baskin (1998) classified eight basic types of MPD. The eight types of MPD are divided into two categories: simple and complex. In seeds with simple MPD, high or moderate (usually 15°C or above) temperatures are required for embryo growth, and in those with complex MPD low (0-10°C) temperatures are required for embryo growth. In our experiments, exposure of *J. dubia* seeds to a sequence of temperatures from warm to cold showed that the embryos began to grow at 25/15°C (Fig. I-4A). Embryos incubated at 25/15°C for 12 weeks grew rapidly to a full length when moved to moderate temperatures (20/10°C and 15/6°C), followed by cold (5°C) temperatures. In natural conditions (Fig. I-2), embryo growth and seed germination were similar to those observed under simulated seasonal temperatures in the laboratory (Fig. I-4A). However, when seeds were initially incubated at 5°C in the temperature sequence, embryo growth was delayed until the exposure to 25/15°C (Fig. I-4B). Thus, moderate temperature (warm stratification) is first required for embryo growth, indicating that seed of *J. dubia* has simple MPD. Low temperature might be necessary for embryo growth because the

embryo kept growing at 5°C after the seeds had been exposed to 25/15°C → 20/10°C → 15/6°C (Fig. I-4). However, we regard that the seeds might respond to widen the range of temperature after warm stratification. In seeds with PD, during progression from dormancy to non-dormancy, the temperature range at which seeds can germinate gradually increases (Baskin and Baskin, 1998). The MPD types can be subdivided further depending on the level of physiological dormancy: non-deep, intermediate and deep. Although GA stimulates the germination of seeds with non-deep and intermediate PD, it does not break deep PD in intact seeds (Nikolaeva, 1977). In seeds with deep PD, GA promotes the growth of embryo, but it does not stimulate germination. GA<sub>3</sub> increased the rate of embryo growth in *J. dubia*, indicating that GA<sub>3</sub> substituted for warm stratification for embryo growth (Fig. I-6A). However, few seeds treated with GA<sub>3</sub> and maintained at 20°C germinated (Fig. I-6B). Therefore, GA<sub>3</sub> did not substitute for the low temperature requirement for dormancy breaking, suggesting that seeds do not have either non-deep or intermediate simple MPD. Since (1) embryos grew at relatively high temperatures for seeds of *J. dubia*, (2) GA<sub>3</sub> only partially overcame dormancy, and (3) warm followed by cold stratification was required to break dormancy, it is confirmed that seeds of this species have deep simple MPD.

The *J. diphylla* (L.) Pers. is the other North American species in the genus *Jeffersonia*. Its seeds have deep simple MPD, requiring high (30/15°C) followed by moderate (20/10 or 15/6°C) temperatures for embryo growth (Baskin and Baskin, 1989). Seeds with fully elongated embryos require pretreatment at low temperature (5°C) for germination. Furthermore, GA<sub>3</sub> can substitute for warm but not for cold stratification in seeds of this species. Thus, the temperature requirements from embryo growth to germination and the effects of GA<sub>3</sub> in *J. dubia* seeds correspond well with those of *J. diphylla*. Distinction

between these two species is shown in the morphology of the leaf shape and capsule opening (Hutchinson, 1920), floral vasculature (Terabayashi, 1981) and pollen exine (Loconte and Estes, 1989). However, it appears that these two eastern Asia-eastern North America disjuncts of *Jeffersonia* exhibit the same dormancy-breaking requirements. Although two species lived on different continents, the climate in these two regions is temperate having four distinct seasons (Li, 1952). They grow on slopes with well-drained soil in rich deciduous forests (Wikipedia, 2013). Seeds of an eastern Asia *Panax* species (Choi, 1977) and those of *Taxus* species (Chien et al., 1998) have deep simple MPD, which is the same type of MPD found in an eastern North American species of *Panax* (Stoltz and Snyder, 1985) and *Taxus* (Nikolaeva et al., 1985), respectively. Therefore, Baskin and Baskin (1989) reported that identical types of MPD in eastern Asian-eastern North American disjuncts that are members of the genera with an Arcto-Tertiary distribution are evidence that this type of dormancy is at least as old as the Tertiary.

Nikolaeva (2001) stated that seeds with MPD come out of dormancy in three stages: (1) PD is broken in the underdeveloped embryo, (2) the underdeveloped embryo grows and becomes fully developed inside the seed, what Nikolaeva called embryo post-development and (3) PD is broken in the seed that now has a fully developed embryo. According to Nikolaeva's formula system (Baskin and Baskin, 2008), this kind of seed dormancy is classified as  $C_{1b}-B-C_3$ . (1) The fresh seeds have underdeveloped embryos with non-deep PD ( $C_1$ ) of the  $C_{1b}$  subtype, thus requiring warm stratification (subscript b) for the dormancy-break. (2) Following the breaking of  $C_{1b}$ , the underdeveloped embryo, now with only MD (B), grows to full size (post-development) at warm temperatures. (3) Finally, the seed with a fully developed embryo germinates after a long period of cold stratification ( $C_3$ ).

Experiments in controlled conditions have shown that a pre-treatment at temperatures (warm stratification) of 25 and 25/15°C for 8 weeks was effective in breaking dormancy (Table I-1). Intermediate temperature pre-treatments (20 and 20/10°C) were not effective in breaking dormancy and resulted in < 50% germination. However, in seeds of *Corydalis solida* with deep simple MPD, incubation at 15/6 and 20/10°C is required to initiate embryo growth while high temperature pre-treatments (23 and 30°C) were ineffective in breaking dormancy (Vandelook and Van Assche, 2009). In nature, although seeds with MPD are exposed to a long period of relatively high temperatures in the summer, the optimum temperature needed to break PD is different between species. The E : S ratio was only 0.471 in seeds incubated continuously at 25/15°C for 52 weeks (Fig. I-3). However, embryo growth was completed at moderate temperatures, after the seeds had been subjected to warm stratification for about 12 weeks (Fig. I-4A). There was variation among species with regard to the timing of embryo growth (Yang et al., 2011): in *Taxus mairei* (Chien et al., 1998), embryo growth occurs during warm stratification; in *J. dubia* and *J. diphylla* (Baskin and Baskin, 1989), embryo growth occurs after warm stratification; and in *Cephalotaxus wilsoniana* (Yang et al., 2011), embryo growth does not occur until after both warm and cold stratification.

GA treatment promoted both the embryo growth and the germination of seeds with non-deep and intermediate simple MPD in some species such as *Chaerophyllum tainturieri*, *Aralia mandshurica* and *Dendropanax japonicum* (Baskin and Baskin, 1998). On the other hand, GA did not promote the germination of seeds with deep simple MPD, for example, *Fraxinus excelsior* (Wcislinska, 1977) *Panax ginseng* (Choi, 1977), *J. diphylla* (Baskin and Baskin, 1989) and *J. dubia* (present study, see Fig. I-6). However, about 70% of *J. dubia* seeds had germinated within 12 weeks when seeds were treated

with  $1000 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> followed by incubation at 15/6°C (Fig. I-7). Following treatment of GA, germination was better at alternating temperature (15/6°C) than at constant temperature (20°C). It is well known that a fluctuating temperature is an absolute requirement for germination of many species (Probert, 1992). In *Cynara cardunculus*, the germination of seeds incubated under the alternating temperature (25/15°C) was promoted compared to under a constant temperature (20°C) (Huarte and Benech-Arnold, 2010). Alternating temperatures broke dormancy of *C. cardunculus* seeds by the increase of GA biosynthesis and a decrease of ABA sensitivity. However, it is different from our case because incubation at 25/15°C after  $1000 \text{ mg} \cdot \text{L}^{-1}$  GA<sub>3</sub> treatment resulted in  $\leq 10\%$  germination. Deep simple MPD contains two parts of PD (Baskin and Baskin, 1998). Embryo cannot be grown until (1) high temperatures (warm stratification) overcome the first part of PD and (2) seeds are subjected to moderate temperatures. After completion of embryo growth, seeds cannot germinate until low temperatures (cold stratification) overcome the second part of PD. The second part of PD was broken by prolonged low temperature (Fig. I-5). It is well known that GA substitutes for warm stratification and stimulates embryo growth (Baskin and Baskin, 1998). We assumed that incubation temperature of 15/6°C played a role of moderate temperature as well as low temperature at the same time. In particular, temperature of 15 and 6°C might act as the moderate temperature for embryo growth and as the subsequent low temperature condition required for completion of radicle protrusion, respectively. However, germination was inhibited when GA-treated seeds were incubated at a constant 5°C. Following the warm stratification, a moderate temperature was also important for embryo growth (Baskin and Baskin, 1998).

Results of this study show that two eastern Asia-eastern North America disjuncts of

*Jeffersonia* have the same type of seed dormancy, despite the fact that they grow in different habitats. The dormant *J. dubia* seed is broken by warm stratification (at 25/15 or 25°C for 8 weeks) followed by cold stratification (at 5°C for 8 weeks). Although seeds of *J. dubia* in nature require about 10–11 months from seed dispersal to germination, seeds can germinate within 3 months with 1000 mg·L<sup>-1</sup> GA treatment accompanied by incubation at 15/6°C. It will allow horticulturalists and seed ecologists to try to speed up the rate of seedling production of seeds with deep simple MPD.

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## CHAPTER II

### **Endosperm Weakening and Endo- $\beta$ -mannanase Activity during Dormancy Breaking of *Jeffersonia dubia* Seeds**

#### ABSTRACT

Seeds of some species have an endosperm which accumulates galactomannan as a storage polysaccharide in the cell walls and endo- $\beta$ -mannanase is involved in hydrolysis of the mannan-rich cell walls during germination and post-germinative seedling growth. Morphology of embryo and endosperm in seeds of *J. dubia* were assessed by light and scanning electron microscopy. Moreover, the temporal and spatial pattern of the development of endo- $\beta$ -mannanase in *J. dubia* seeds was also investigated. The hydrolytic products of endosperm cell walls in seeds of *J. dubia* were mainly composed of mannose, and smaller quantities of glucose and galactose, indicating that the endosperm contained mannan-rich cell walls. After seeds were buried in May, endo- $\beta$ -mannanase was not detected for three months when underdeveloped embryo hardly grew at all. The embryo started to grow after September and the activity of endo- $\beta$ -mannanase increased in micropylar endosperm regions. The erosion of the endosperm cell wall was observed at the lateral side around embryo while the micropylar endosperm appeared to be no obvious signs of being collapsed or damaged. The increase of enzyme activity

coincided with the increase in length of the embryo under moderate temperatures. Embryo stopped growing during winter season for about 2-3 months and enzyme activity was also low state. However, enzyme activity increased again during germination occurred in early March. Micropylar endosperm rupture occurred and germination was completed. The seasonal pattern of endo- $\beta$ -mannanase activity was unique in seeds with deep simple MPD and different with that of PD, MD, or non-deep MPD.

Keywords: endo- $\beta$ -mannanase, endosperm weakening, lateral endosperm, micropylar endosperm

## INTRODUCTION

Mannans or glactomannans are the major components of endosperm cell walls and function as reserve carbohydrates in seeds of lettuce, fenugreek, date and many other species (Matheson, 1984; Halmer, 1985). Although mannan polysaccharides play a role as a carbohydrate source to support germination and the growth of the seedling, they have also been demonstrated to modulate the mechanical strength of the endosperm of seeds to facilitate radicle protrusion (Groot and Karssen, 1987). In seeds whose embryos are embedded in a rigid endosperm, the weakening of the mechanical restraint of the endosperm cells is a prerequisite for radicle protrusion (Groot and Karssen, 1987). Weakening of endosperm is involved in cell wall hydrolysis (Watkins et al., 1985).

Three enzymes are involved in degradation of the endosperm through the hydrolysis of galactomannans and mannans in cell walls:  $\alpha$ -galactosidase, endo- $\beta$ -mannanase and  $\beta$ -mannosidase (Reid and Meier, 1972; McCleary and Matheson, 1976; Buckeridge and Dietrich, 1996). Endo- $\beta$ -mannanase is endohydrolase that catalyses the cleavage of  $\beta(1\rightarrow4)$  bonds in the mannan backbone;  $\beta$ -mannosidases and  $\beta$ -glucosidases are exohydrolases that attack the non-reducing end of the mannan polymer, releasing mannose or galactose units, respectively (Moreira and Filho, 2008; van Zyl et al., 2010). Endo- $\beta$ -mannanase is considered the major enzyme for the degradation of mannan-containing polymers present in cell walls (Bewley, 1997). Mannohydrolase and  $\alpha$ -galactosidase are incapable of releasing mannose or galactose from native cell walls without prior cleavage by endo- $\beta$ -mannanase (Bewley et al., 1983).

Endo- $\beta$ -mannanase has been studied extensively in relation to seed germination of

many species, such as tomato (Groot and Karssen, 1987; Toorop et al., 1996), pepper (*Capsicum annum*; Watkins and Cantliffe, 1983), tobacco (Leubner-Metzger et al., 1995), melon (*Cucumis melo*; Welbaum et al., 1995), *Datura ferox* (Sanchez et al., 1986), *Lepidium sativum* (Müller et al., 2006), and *Genipa americana* (Queiroz et al., 2012). However, most of these seeds have fully developed embryos, which do not have MD or MPD. Moreover, some seeds have non-deep PD so that its dormant period was relatively short compared to those have deep PD. Seed dormancy of *J. dubia* has been classified as deep simple MPD (Rhie et al., 2014). Very little is known about the relationship between endo- $\beta$ -mannanase activity and dormancy break in seeds with MPD.

The purpose of this study was to investigate how the cell wall hydrolic enzyme expressed in seed with a longer period of dormancy, such as seeds of *J. dubia*. The sugar composition analysis and anatomical characterization of the cell walls during dormancy breaking of *J. dubia* was also investigated.

## MATERIALS AND METHODS

### Seeds

Seed capsules of *J. dubia*, consisting of 10–15 seeds, were harvested from a population of *J. dubia* in Yongin (37°05' N, 127°24' E), Geonggi, Korea on 22 May 2011. Here, *J. dubia* grows in a mesic deciduous woodland in Hantaek Botanical Garden that is an *ex situ* conservation habitat. Seeds were removed from the capsules by hand, winnowed and then air-dried at room temperature (22–25°C) for 4 days. The dried seeds were packed in sealed impermeable plastic bags and stored at 5°C for 4 days until the beginning of the experiments.

### Cell wall sugar composition

To assay the sugars in cell wall, three replicates of 80 seeds allowed imbibing in water for two days. After the testae were removed from seeds with forceps and razor blades, the testa and endosperm were lyophilized and ground in liquid nitrogen to a fine powder. Powdered samples were washed with 1 ml 80% ethanol three times to remove soluble sugars and dried at 80°C. Cell walls were extracted using the method described by Dahal et al. (1997) with slight modifications. The samples were washed sequentially with 1 mL each of water, 50mM NaCl, methanol, acetone (two times), 50mM NaCl, and water, and dried. The dry samples (1.0–1.5 mg) with three replications each were hydrolyzed cell walls to sugar monomers with 200  $\mu$ L of 72% H<sub>2</sub>SO<sub>4</sub> for 1 h at room temperature, then

neutralized with sodium hydroxide. The extracts were filtered through 0.45 µm nylon filter (Acrodisc® 13 mm Syringe Filter, Pall Co., Washington, NY, USA). Sugars were analyzed by a HPLC (DX-2500, Dionex, Sunnyvale, CA, USA) connected to an ED40 electrochemical detector and CarboPac PA1 (4×250mm, Dionex) column. Potassium hydroxide was used as the mobile phase at a flow rate of 1.0 ml/min. Mannose, glucose, galactose, arabinose, xylose standards were injected as references.

### **Anatomical characterization of the endosperm**

The lateral and micropylar endosperm of *J. dubia* seeds were examined by scanning electron microscopy. The seeds were imbibed in distilled water for 5 days and cut transversally. Seeds were fixed in a Karnovsky fixative (5% glutaraldehyde + 4% formaldehyde in 0.1M cacodylate buffer + 50mg CaCl<sub>2</sub>) for two hours at 5°C. After fixation seeds were washed three times with 0.05M sodium cacodylate buffer for 10 minutes, and post-fixed in 2% osmium tetroxide for 2 h. The samples were washed briefly in distilled water and then dehydrated in ascending solutions of ethanol (30, 50, 70, 80, 90, and 100%, 10 min each). Dehydrated samples were critical-point dried. The stubs were wrapped in aluminum foil, sputter coated with gold and observed in a scanning electron microscope (Leo Evo 40, Carl Zeiss, Wetzlar, Germany) using a 5 kV acceleration voltage and 10 µA beam current. The morphological and anatomical parameters evaluated in the micropylar endosperm were thickness (number of cell layers), cell diameter and cell wall thickness and in the lateral endosperm cell diameter and cell wall thickness. The thickness of the micropylar endosperm was measured in six seeds. The mean diameter of the cells was derived from two measurements on 10 cells from

three seeds. For measurement of the average thickness of the cell walls 10 cells in three seeds were used. The assessment of these parameters was done from the scanning microscope images.

### **Structural changes in the micropylar and lateral endosperm**

Ten seeds were exhumed on 22 May and 6 October 2011, respectively, and were fixed in 2.5% formaldehyde and 0.05 M sodium cacodylate buffer at 5°C. Seeds were longitudinally sectioned with razor blade and observed under field-emission scanning electron microscope (SUPRA 55VP, Carl Zeiss, Wetzlar, Germany) using a 15 kV acceleration voltage, respectively.

### **Embryo growth and germination in a field condition**

Embryo growth in seeds of *J. dubia* was monitored under field conditions by burying seeds and exhuming them at set time intervals. Ten nylon bags were filled with 20 seeds each and 10 g of white sand. These bags were buried at a depth of 3 cm in an experimental garden in the campus of Seoul National University, Seoul, Korea on 30 May 2011. The soil temperature at a depth of 3 cm was measured every 30 minutes with a thermo data logger (Watch Dog Model 450, Spectrum Technologies, Inc., Plainfield, IL, USA), and the weekly maximum and minimum temperatures were calculated. On the 6<sup>th</sup> day of each month, ten seeds were removed at random from one nylon bag and the embryo length was measured. Seeds were cut in half under a dissecting microscope using a razor blade, and the embryo length was measured with an ocular micrometer from 6

June 2011 to 6 February 2012. The ratio of embryo length to seed length (E:S ratio) was calculated. Photographs of seeds were taken with a digital microscope (MV200UV, Cosview Technologies Co., Shenzhen, China). The timing of seed germination under field conditions was determined. Three nylon bags were filled with 100 seeds each and buried approximately 1-cm-deep in an experimental garden in the campus of Seoul National University. Seeds with an emerged radicle were counted and removed weekly for 1 year. Germination was defined as radicle protrusion and elongation of more than 2 mm. Intact seeds that had not germinated were buried again.

#### **Endo- $\beta$ -mannanase extraction and assay**

Ten seeds, collected monthly in a field conditions from 6 June 2011 to 6 March 2012, were used to measure endo- $\beta$ -mannanase activity in the endosperm of *J. dubia*. Seeds were divided into two parts to produce the micropylar endosperm region (about one-fifth the length of seed) and lateral endosperm region (about four-fifth the length of seed). Endosperms were ground in 1.0 mL of K-phosphate buffer (0.2 M, pH 6.8) in a mortar and pestle. After centrifugation at 10,000 g for 5 minutes, the supernatants were used to assay endo- $\beta$ -mannanase activity. Enzyme activity was determined with an activity gel of 8 mm thickness, containing 0.5% (w/v) of locust bean gum, McIlvaine buffer (0.05 M citric acid/0.1 M Na<sub>2</sub>HPO<sub>4</sub>, pH 5.0), and 0.8% of Phytogel (Sigma) on Petri plates. Ten microliters of extract were applied to holes of 2 mm diameter made in the gel.

After application the gel was incubated at 40°C for 16 hours in the dark, and then washed in McIlvaine buffer (pH 5.0) for 30 minutes, stained with 0.5% (w/v) Congo Red for 15 minutes, washed with 96% ethanol for 10 minutes, and destained in 1 M NaCl for

6 hours. All staining and destaining steps were performed on a rotating platform.

Commercial endo- $\beta$ -mannanase from *Aspergillus niger* (Megazyme, North Rocks, Sydney, Australia), which was diluted from 69.67 nkat to 0.006967 nkat, was used to develop a standard curve. Enzyme activity of the extracts from the micropylar or lateral endosperm was calculated from standard curves using regression analysis of the diameters of the hydrolyzed arrears versus the log of the endo- $\beta$ -mannanase enzyme activity.

### **Localization of endo- $\beta$ -mannanase**

Fifteen seeds, collected monthly in a field conditions, were used for localization of endo- $\beta$ -mannanase activity. The integument was removed by tweezers, and the seeds were rinsed in deionized water and cut in half with a razor blade. Seed parts were blotted dry on filter paper and laid on top of an activity gel (2 mm thick), cut side down. Activity gels were incubated for 1 hour at room temperature. Seed parts were then removed from the gel with tweezers. After incubation, the gels were stained and destained, as described above.

### **Statistical analysis**

Data was subjected to statistical analysis (ANOVA), where the averages of sugar composition were compared with Tukey test at the level of 5% probability, using the SAS system for window V8 (SAS Inst. Inc., Cary, NC, USA).

## **RESULTS**

### **Cell wall sugar composition**

Testa and endosperm cell walls of seeds were isolated from storage polysaccharides (starch and fructans) and chemically hydrolyzed. Mannose, glucose, galactose, arabinose, and xylose were detected (Table II-1). In endosperm cell walls, mannose is the most abundant neutral sugar, accounting for about 56.7% of total sugar. The second most abundant sugar is glucose (22.8%) followed by arabinose and galactose. The ratio mannose/galactose is 7. In contrast, the testa cell walls were composed primarily of glucose and arabinose, with relatively small amounts of galactose, mannose and xylose.

### **Anatomical characterization of the endosperm**

The testa appeared firmly attached to the endosperm (Fig. II-1A). The micropylar endosperm consisted of six to eight cell layers (Fig. II-1B), which together corresponded to an average thickness of 93.5  $\mu\text{m}$  (Table II-2). The cells of the lateral endosperm appeared as having a rounded to polygonal shape (Fig. II-1C) with an average diameter of 44.1  $\mu\text{m}$  and cell wall thickness of 0.6  $\mu\text{m}$  (Table II-1), values significantly higher than those of the micropylar endosperm. The cells of the micropylar endosperm appeared as flat, elongated and with a polygonal shape (Fig. II-1D) with an average (longest) diameter of 12.0  $\mu\text{m}$  and cell wall thickness of 1.0  $\mu\text{m}$  (Table II-1).

Table II-1. Sugar composition of polysaccharides from cell walls of endosperm and testa of *Jeffersonia dubia* seeds. The values represent the mean  $\pm$  SD of three samples.

Sugar	Endosperm		Testa	
	$\mu\text{g}\cdot(80 \text{ endosperm})^{-1}$	% of total sugars	$\mu\text{g}\cdot(80 \text{ testa})^{-1}$	% of total sugars
Mannose	$2279.8 \pm 117.9$	$56.7 \pm 1.5$	$78.1 \pm 16.4$	$4.9 \pm 0.7$
Glucose	$920.5 \pm 152.8$	$22.8 \pm 2.2$	$903.2 \pm 35.9$	$56.7 \pm 0.8$
Arabinose	$461.2 \pm 29.9$	$11.4 \pm 0.3$	$351.6 \pm 13.7$	$22.1 \pm 0.4$
Galactose	$326.0 \pm 8.6$	$8.1 \pm 0.4$	$185.3 \pm 18.6$	$11.6 \pm 0.9$
Xylose	$39.1 \pm 9.7$	$1.0 \pm 0.3$	$75.0 \pm 11.5$	$4.7 \pm 0.6$

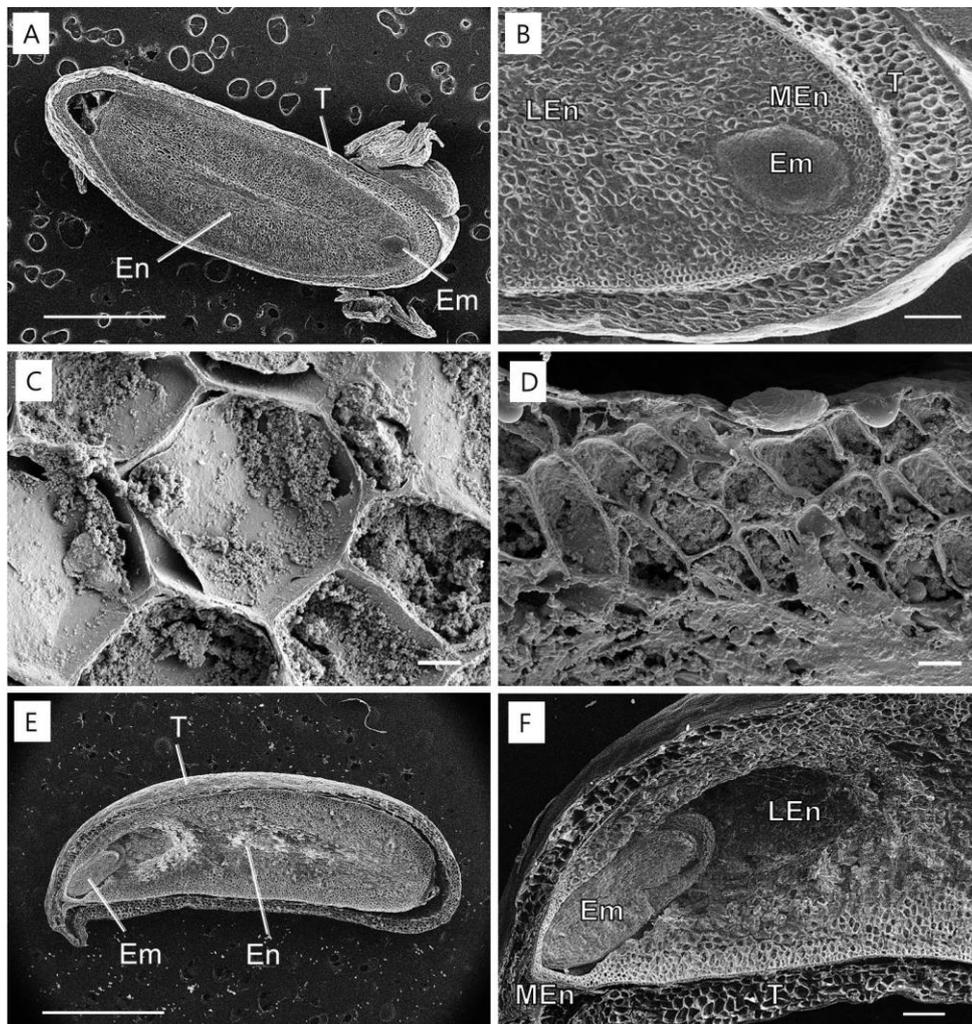


Fig. II-1. Scanning electron microscopy of *Jeffersonia dubia* seeds: General view of the embryo (Em), endosperm (En) and testa (T) at the seed dispersal time (A); cells of the lateral endosperm (LEn), micopylar endosperm (MEn), and embryo (Em) at dispersal time (B); detailed view of the micopylar endosperm (C) and lateral endosperm (D); general view of the embryo (Em), endosperm (En), and testa (T) (E); and cells of the lateral endosperm (LEn), micopylar endosperm (MEn), and testa (F). The bar in Fig. II-1A, E is 1 mm, in Fig. II-1B, F 100  $\mu\text{m}$  and Fig. II-1C, D 10  $\mu\text{m}$ .

Table II-2. Morphometric parameters of the micropylar and lateral endosperm of *Jeffersonia dubia* seeds. Averages followed by the same lower case and uppercase do no differ by the Tukey test at 5% level of probability.

Endosperm	Parameters	Average $\pm$ SD ( $\mu\text{m}$ )
Micropylar	Endosperm thickness	93.5 $\pm$ 6.1
	Cell diameter	12.0 $\pm$ 4.5 A
	Cell wall thickness	1.0 $\pm$ 0.1 a
Lateral	Cell diameter	44.1 $\pm$ 10.0 B
	Cell wall thickness	0.6 $\pm$ 0.1 b

### **Embryo growth and germination in a field condition**

The average E:S ratio ( $\times 10^2$ ) of freshly matured seeds was  $9.0 \pm 0.2$  (mean  $\pm$  SE) (Fig. II-2 and II-3A). Significant growth of the embryo started between 6 September and 6 October 2011 (Fig. II-2). The embryos grew rapidly between 6 October and 6 December 2011, when mean maximum and minimum temperatures were 20 and 0°C, respectively. Growth of the embryos slowed until 6 January and reached the critical E:S ratio ( $\times 10^2$ ) ( $96.0 \pm 3.0$ ) required for germination. However, the first germination was recorded on 13 February 2012 and about 5% of the seeds sown at a depth of 1 cm germinated. Mean maximum and minimum temperatures were 5.6 and -0.8°C, respectively. Peak germination of seeds occurred when temperatures started rising in late February. By beginning of March 2012, 86.7% of the seeds germinated.

### **Structural changes in the micropylar and lateral endosperm**

In May 2011, at the time of seed dispersal, the embryo was underdeveloped (Fig. II-1A and II-3A). On 6 October 2011, sharp differences were seen in lateral endosperm (Fig. II-1E, F). The lateral endosperm appeared eroded; in many area of wall material seemed to be missing and the contours of the cell underneath were clearly discernible. Even when the loss of lateral endosperm was extensive, the micropylar endosperm appeared to show no obvious signs of collapse or damage. Fig. II-3F shows that micropylar endosperm rupture occurred and germination was completed in March 2012.

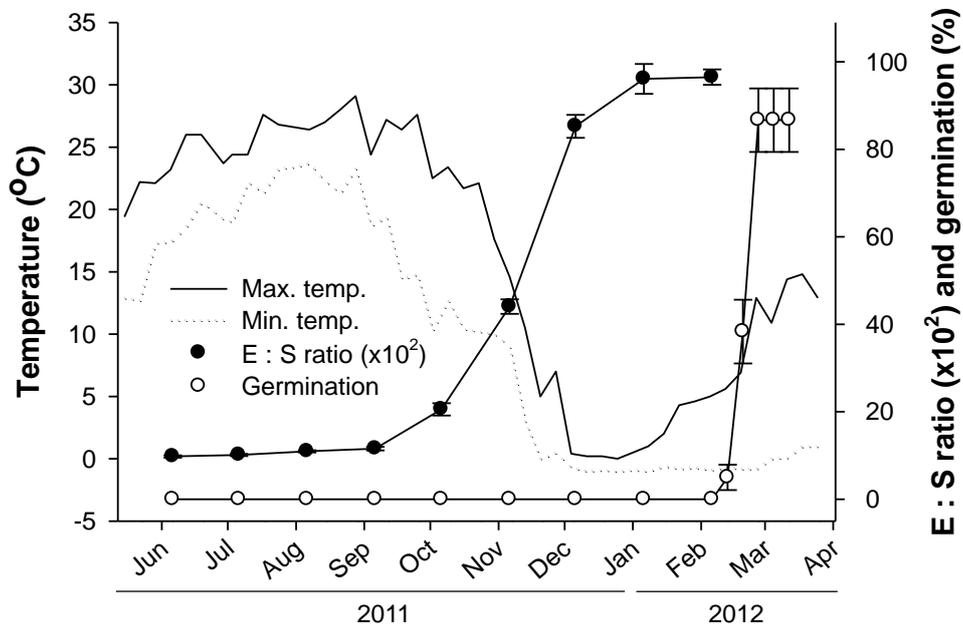


Fig. II-2. Mean weekly minimum and maximum soil temperatures, phenology of embryo growth and germination in *Jeffersonia dubia* seeds sown in soil on 30 May 2011.



Fig. II-3. Embryo (arrow in A) growth in seeds of *Jeffersonia dubia* outdoors in Seoul, Korea: R, radicle; En, endosperm; T, testa.

### **Endo- $\beta$ -mannanase activity**

Endo- $\beta$ -mannanase was not detected for three months from June to August (Fig. II-4). Enzyme activity was only detected in the micropylar endosperm region in September, when the embryo was still small. The activity of endo- $\beta$ -mannanase increased in October and observed in both micropylar and lateral endosperm regions. In November, enzyme activity was higher in the lateral endosperm region than in the micropylar. However, enzyme activity decreased in December onward and maintained the low level of expression from January to February. In March, germinated seeds showed high activity of endo- $\beta$ -mannanase in both micropylar and lateral endosperm regions.

Tissue printing was used to locate endo- $\beta$ -mannanase activity in the endosperm during dormancy breaking under field conditions. The activity first appeared in the micropylar endosperm region in September and subsequently spread throughout the whole endosperm until November (Fig. II-5). In November, seed showed high enzyme activity in the lateral endosperm but low in the micropylar (Fig. II-5D). The activity in the micropylar and lateral endosperms increased again in March and coincided with radicle protrusion (Fig. II-5G).

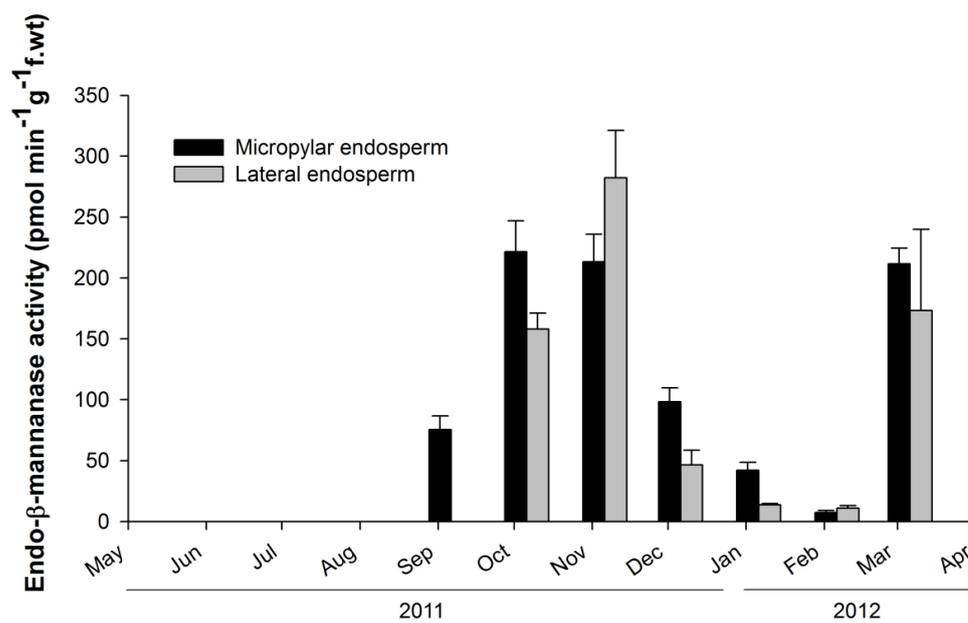


Fig. II-4. Endo-β-mannanase activity in micropylar and lateral endosperm of *Jeffersonia dubia* seeds under a field condition from June 2011 to March 2012, when the radicle protruded. Bars are ±1 SEs.

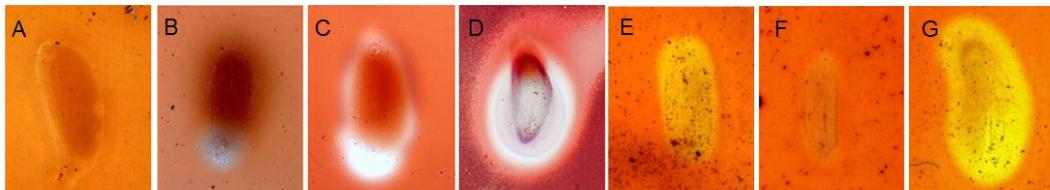


Fig. II-5. Tissue printing of longitudinally-cut *Jeffersonia dubia* seeds during the dormancy breaking, showing endo- $\beta$ -mannanase activities as clearings in micropylar and lateral endosperm in May (A), September (B), October (C), November (D), December (E), February (F), and March (G).

## DISCUSSION

The cell wall storage polysaccharides have been usually classified into three groups: mannans, xyloglucans and galactans (Reid and Edwards, 1995). In *J. dubia* seeds, the occurrence of relatively large quantities of mannose and smaller quantities of galactose in the hydrolysate also indicates that mannans, possibly galactomannans, are the main components of these cell walls (Table II-1). Glucose which is also present in considerable quantities in the hydrolysate, might originate both from cellulose and possibly also from glucose units in the mannan chains (Groot et al., 1988). Galactomannans are composed of a linear backbone of  $\beta$ -(1 $\rightarrow$ 4)-linked-D-mannose residues to which single units of D-galactose residues are attached by  $\alpha$ -(1 $\rightarrow$ 6)-linkages, providing polymers with different mannose:galactose ratios (Dea and Morrison 1975). The mannose:galactose ratio varies from 1:2 to 20:1; the amount of galactose residues in the mannan polymer influences its solubility, viscosity, and interactions with other cell wall polysaccharides; as the mannose:galactose ratio diminishes, the solubility of the polymer increases, by preventing the formation of insoluble paracrystalline structures among the mannan chains. The mannose:galactose ratio in galactomannans of *J. dubia* is an average of ratio of 7:1 (Table II-1), and their values are similar with that of tomato, lettuce, or *Datura* species (Table II-1; Dahal et al., 1997; Sánchez et al., 1990; Halmer and Bewley, 1979).

The micropylar endosperm cells appeared to be predisposed for radicle protrusion because its cells were smaller with less thick cell walls than cells of the lateral endosperm (Gong et al. 2005). This predestined region of micropylar endosperm was also observed for the seeds of *S. lycocarpum* (Pinto et al. 2005) and *C. arabica* (da Silva et al. 2004).

Although in *J. dubia* the lateral endosperms were bigger than the micropylar endosperm (Fig. II-1), cell walls of lateral endosperm were thinner than those of micropylar endosperm (Table II-2). The number of cell layers of the micropylar endosperm of *J. dubia* was similar as compared to coffee, which has three to four cell layers (da Silva et al. 2004).

Ripe seeds of *J. dubia* had an underdeveloped embryo which hardly grew for 3 months (June-September) (Fig. II-2). Endo- $\beta$ -mannanase activity was also not detected in both the micropylar and the lateral endosperm from June to August (Fig. II-4 and II-5). However, enzyme activity increased in micropylar endosperm region in September prior to the initiation of embryo growth. We considered that this enzyme activity of micropylar endosperm region was not that of the exact micropylar endosperm but that of the lateral endosperm. The micropylar endosperm region was prepared by one-fifth the length of seed so that micropylar endosperm region might contain large amounts of lateral endosperm. Carrot (*Daucus carota* L.) and *Annona crassiflora* seeds have underdeveloped embryo and the initial occurrence of endo- $\beta$ -mannanase activity in micropylar endosperm region, followed by localization in the lateral endosperm, coincided with the beginning of embryo growth (Homrichhausen et al., 2003; da Silva et al., 2007).

Prior to the completion of seed germination, endo- $\beta$ -mannanase activity increases in the micropylar endosperm of *Datura ferox* (Sánchez et al., 1986) and tomato (Toorop et al., 1996). However, endo- $\beta$ -mannanase activity in seeds of lettuce, celery, date, Chinese senna, pepper, and some legume species increases only after the completion of germination. In monocotyledonous seeds, endo- $\beta$ -mannanase activity has been detected

after the completion of germination in the endosperm of date palm (Gong et al., 2005) and rice (Ren et al., 2008). In *J. dubia*, the enzyme had minimal activity in either the lateral or micropylar endosperm prior to the completion of germination. Enzyme activity significantly increased after radicle protrusion. Thus the emergence of the radicle from the seed in *J. dubia* is unlikely to be dependent upon the weakening of the mannan-rich components of the cell walls of the surrounding endosperm. There is the possibility that other hemicellulases which degrade the minor components of the cell walls play a role in germination, although this would seem to be less likely (Gong et al. 2005). Whether enzyme activity was observed before or after germination, it was regarded as important for the mobilization of galactomannan reserves to support the early growth of seedlings (Bewley, 1997; Gong et al., 2005).

Endo- $\beta$ -mannanase activity was increased by the dormancy breaking treatments through cold stratification (Downie et al., 1997), dark treatment (Goggin et al., 2011), and priming (Nascimento et al., 2001; Kim and Kim, 2010). However, in these previous researches, once endo- $\beta$ -mannanase activity was initiated this enzyme activity increased continuously until germination. In *Annona crassiflora* seeds with non-deep simple MPD, after undergoing the period of stratification, endo- $\beta$ -mannanase activity increased steadily together with an increase in embryo size until germination (da Silva et al., 2007). In carrot seeds with MD, endo- $\beta$ -mannanase activity was first detected in the endosperm after 18 h from the start of imbibition, and it increased about fivefold at germination (at around 48 h) (Homrichhausen et al., 2003). On the other hand, *J. dubia* seeds with deep simple MPD, the enzyme fluctuated in activity over a wide range of time scale during warm or cold stratification. Endo- $\beta$ -mannanase activity of *J. dubia* was not detected during warm stratification (about 3 months). The increase of enzyme activity in the

endosperm coincided with the increase in embryo length within the seed under moderate temperatures (October-November). During winter season for about 2-3 months, seeds had been shown in a state of quiescence and enzyme activity was also low. However, enzyme activity increased again after germination occurred in early March. This seasonal pattern of enzyme activity was different with that of PD, MD, or non-deep MPD.

The phenology and temperature requirements for embryo growth and seed dormancy break in *J. dubia* are similar to those of *J. diphylla* (Berberidaceae), *Panax ginseng* (Araliaceae), *Cardiocrinum cordatum* (Liliaceae), and *Cephalotaxus wilsoniana* (Cephalotaxaceae) although these seeds belonging to different plant families differ in the timing of seed maturation/dispersal (Kondo et al., 2006). These seeds belong to deep simple MPD, in which they require high (e.g., summer or 25/15°C) followed by moderate (e.g., autumn or 20/10 or 15/6°C) temperatures for embryo growth (Rhie et al., 2014). Seeds with fully elongated embryos must be subjected to low temperature (e.g., winter or 5°C) for radicle emergence. The pattern of endo- $\beta$ -mannanase activity of other seeds with deep simple MPD may be similar with that of *J. dubia* because they also need warm stratification for embryo growth and cold stratification for radicle protrusion. This finding seemed to be the first report that revealed the pattern of endo- $\beta$ -mannanase activity in seeds with deep simple MPD which is one of the phylogenetically basal angiosperms. The seeds of ash (*Fraxinus excelsior*) have deep simple MPD (Nikolaeva, 1967; Finch-Savage and Clay, 1997), which was the same type of dormancy as in *J. dubia*. Warm stratification is required for the break of dormancy in the small but fully differentiated embryo, and subsequent cold stratification is required for germination (Finch-Savage and Leubner-Metzger, 2006). Within the dormant seed population there is a distribution of forces required to puncture the endosperm layer covering the radicle, which moves to

lower puncture forces during stratification, suggesting that these changes begin to occur while the seed is still dormant. We observed that the timing of endo- $\beta$ -mannanase generation coincided with pronounced erosion of the endosperm cell wall (as shown by SEM observations, Fig. II-1). Such change in cell wall structures might be induced as a result of digestion of cell wall galactomannan by endo- $\beta$ -mannanase that develops prior to embryo growth in the lateral endosperm. Therefore, we suppose that endo- $\beta$ -mannanase facilitates endosperm weakening of seeds and decreasing puncture force by breaking intercellular adhesion during warm or cold stratification.

In summary, *J. dubia* seeds with both morphological and physiological dormancy could germinate after long period of warm and cold stratifications. Thus it appears that for embryo growth and germination to be completed, if the walls of the endosperm cells are thickened, there is likely to be the requirement for the participation of cell wall hydrolases, including endo- $\beta$ -mannanase to permit penetration by radicle. The increase of enzyme activity in the endosperm coincided with the increase in embryo length within the seed. Although enzyme activity decreased in winter season when embryo ceased to grow, it increased again during germination in spring season. The pattern of endo- $\beta$ -mannanase during dormancy breaking in seeds with deep level of MPD dormancy was first observed in this study.

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## CHAPTER III

### **Gibberellic Acid and Abscisic Acid Involved in Embryo Growth and Germination of *Jeffersonia dubia***

#### ABSTRACT

Many studies support that the innate balance of GA and ABA biosynthesis and catabolism determined whether a seed would remain dormancy or germinate. However, few studies have been carried out whether GA-ABA balance could control the embryo growth and germination in seeds with morphophysiological dormancy (MPD). We hypothesized that high GA/ABA ratio broke the first part of physiological dormancy (PD) for embryo growth and the second part of PD for radicle protrusion while low GA/ABA maintain both the first and the second PD. In these experiments, we showed that exogenous GA and fluridone, ABA biosynthesis inhibitor, treatments promoted embryo growth in seed of *Jeffersonia dubia*, but exogenous ABA restrained the growth of embryo. When seeds were exhumed in November, embryo in seeds was fully grown but dormant. Although these seeds required cold stratification, exogenous GA and fluridone could substitute cold stratification and promote the radicle protrusion. However, ABA or paclobutrazol, GA biosynthesis inhibitor, treatments suppressed seed germination though seeds were subjected to cold stratification for 8 weeks. The data support a GA-ABA hormone

balance mechanism could be applied to seeds with deep simple MPD as well as non-deep PD or non-dormancy. We also measured the ABA contents of seeds during the season. Endogenous ABA content was relatively high in seeds freshly harvested. However, the ABA content decreased approximately 8-fold following warm and cold stratification.

Keywords: abscisic acid, embryo growth, gibberellic acid, hormone balance theory

## INTRODUCTION

Seed dormancy and germination were regulated by plant hormones (Hilhorst, 1995). According to the revised hormone-balance hypothesis, ABA and GA act independently at different times and sites in the seed life (Karssen and Laçka, 1986). ABA only induces dormancy during seed development and excluded its role during imbibition and dormancy maintenance while GA plays a role in dormancy break and in the promotion of germination.

However, more recent studies showed that GA and ABA can act at the same time on dormancy and germination (Finch-Savage and Leubner-Metzger, 2006). Inhibition of GA biosynthesis during seed development mimics the effects of exogenous ABA, for example in suppressing vivipary which is precocious germination during seed development. In *Arabidopsis* Cvi, when the non-dormant seeds started to germinate, the ABA content of the dormant seeds is two times higher than that of non-dormant seeds. These results strongly suggest that the maintenance of dormancy in imbibed seeds is regulated by active synthesis of ABA. Added GA to the seeds inhibited ABA biosynthesis and accelerated the rate of germination (Grappin et al., 2000). On the other hand, GA+ABA nullified the effect of promoting the germination. In coffee, ABA prevents endosperm weakening during seed germination but fluridone, have been used in experiments to inhibit ABA biosynthesis, treatment accelerates radicle protrusion of coffee seeds (da Silva et al., 2004). Recently, a genome-wide analysis of the *Arabidopsis* revealed that the innate balance of GA and ABA biosynthesis and catabolism determined whether a seed would remain dormancy or germinate (Cadman et al., 2006). While dormancy maintenance depends on low GA/ABA ratios, dormancy break involves in high GA/ABA

ratios through the increased GA biosynthesis and ABA degradation (e.g. Ali-Rachedi *et al.*, 2004; Cadman *et al.*, 2006). However, experiments with other species are needed to determine whether this is a general phenomenon (Finch-Savage and Leubner-Metzger, 2006).

Deep simple MPD contains two parts of PD (Baskin and Baskin, 1998). Embryo cannot be grown until (1) high temperatures (warm stratification) overcome the first part of PD and (2) seeds are subjected to moderate temperatures. After completion of embryo growth, seeds cannot germinate until low temperatures (cold stratification) overcome the second part of PD. The second part of PD was broken by prolonged low temperature. In some species exhibited deep simple MPD such as *Taxus mairei*, *Fraxinus excelsior*, and *Styrax japonicus*, the freshly-harvested seeds contain a high concentration of ABA and are dormant (Chien *et al.*, 1998; Finch-Savage and Leubner-Metzger, 2006; Horimoto *et al.*, 2011). These underdeveloped embryos continue to grow during warm stratification and ABA concentrations decrease progressively. On the other hand, significant GA<sub>1</sub> concentrations did not occur during warm stratification, and just GA<sub>3</sub> was detected in a short period during embryo growth (Finch-Savage and Leubner-Metzger, 2006). After embryo had grown to the full length of the seed, subsequent cold stratification was required for germination and was associated with an increase in GA concentration (Finch-Savage and Leubner-Metzger, 2006; Horimoto *et al.*, 2011). It indicated that endogenous GA and ABA is closely related to dormancy breaking in seeds with deep simple MPD. However, there is insufficient evidence that GA/ABA balance controls the maintenance and break of dormancy. Thus, this study investigated whether the GA/ABA balance determine on seed dormancy and break of *J. dubia*. Thus, it will get a better understanding in GA/ABA balance role in basal angiosperm.

## **MATERIALS AND METHODS**

### **Collection and storage**

*J. dubia* seeds were harvested on 28 May 2010 and 22 May 2011 in deciduous woodland of Hantaek Botanical Garden. Seeds were winnowed and air-dried at room temperature (22–25°C) for 2–4 days. Seeds not used immediately in the experiments were put into paper envelopes at 5°C until they were used.

### **Germination test**

A treatment consisted of three replicates of 30 seeds each. At each observation, seeds with an emerged radicle were recorded and removed from the dishes; water was added as needed to keep the seeds moist. The experiments were terminated at which time no additional germination had occurred for 3 weeks.

### **Effect of exogenous GA and ABA on embryo growth**

On 6 June 2011, seeds were soaked for 24 h in distilled water, 1000 mg·L<sup>-1</sup> GA<sub>3</sub> (Duchefa, Haarlem, The Netherlands), and in combinations of four concentrations (10, 100, 500, and 1000 mg·L<sup>-1</sup>) of ABA (Sigma, St Louis, MO, USA) and 1000 mg·L<sup>-1</sup> GA<sub>3</sub>. After treatments, seeds treated with 500 mg·L<sup>-1</sup> Benomyl for 3 h for bacterial control, and then rinsed in distilled water. Seeds were placed in plastic Petri dishes on two layers of filter paper moistened with distilled water. Petri dishes were sealed with Para film (Pechiney Plastic Packaging, Menasha, WI, USA) to retard water loss during incubation.

Seeds were incubated in light- and temperature-controlled incubators at 20/10°C (12/12 h) with 12 h of fluorescent light (photosynthetically active radiation 30–40  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) during the high temperature period of the regime. After 12 weeks, 30 healthy seeds were extracted from each treatment. Embryos were excised with razor blade and their lengths measured using a dissecting microscope equipped with a micrometer.

### **Effect of GA and fluridone on germination**

On 6 June 2011, seeds were soaked for 24 h in distilled water, 1000  $\text{mg}\cdot\text{L}^{-1}$  GA<sub>3</sub>, three concentrations (10, 100, and 500  $\text{mg}\cdot\text{L}^{-1}$ ) of fluridone (Duchefa, Haarlem, The Netherlands), and in combinations of these compounds. Germination was recorded weekly, and germination percentages were calculated.

### **Effect of ABA and paclobutrazol on germination in warm stratified seeds during cold stratification**

On 2 June 2010, seeds were placed in fine-mesh polyester bags and buried at a depth of 3 cm in an experimental garden on the campus of Seoul National University, Seoul, Korea. After 5 months, on 10 November 2010, seeds were removed from their bag. The average ratio of embryo length to seed length (E:S ratio) of these seeds was  $0.675 \pm 0.012$  (mean  $\pm$  SE,  $n = 10$ ). These seeds were dried slightly in the laboratory at room temperature (22–25°C) for 6 h and were soaked for 24 h in distilled water, 100  $\text{mg}\cdot\text{L}^{-1}$  paclobutrazol (Duchefa, Haarlem, The Netherlands), and 100  $\text{mg}\cdot\text{L}^{-1}$  ABA. After treatments, seeds were subjected to the low temperature of 5°C for 12 weeks (cold

stratification), and then all seeds were transferred to 20/10°C and checked germination. Control seeds imbibed with distilled water were incubated at 20/10°C without cold stratification.

### **Effect of GA<sub>3</sub> and fluridone on germination in warm stratified seeds**

This study determined whether GA<sub>3</sub> and fluridone could substitute to the cold stratification in warm stratified seeds. Seeds were sown in an experimental garden on 2 June 2010 and exhumed from the field on 10 November 2010. These seeds were kept for 6 h in the laboratory at room temperature and soaked in distilled water, three concentrations (10, 100, and 1000 mg·L<sup>-1</sup>) of GA<sub>3</sub>, 100 mg·L<sup>-1</sup> fluridone, and 1000 mg·L<sup>-1</sup> GA<sub>3</sub> + 100 mg·L<sup>-1</sup> fluridone.

### **Endogenous hormone (ABA) analyses**

To detect the endogenous hormones during breaking dormancy, seeds were harvested on 22 May 2011 in deciduous woodland of Hantaek Botanical Garden. On 27 May 2011, each of the 12 fine-mesh polyester bags filled with 60 seeds and white sand was buried at a depth of 3 cm in an experimental garden in the campus of Seoul National University, Seoul, Korea. Each of three bags of seeds were exhumed from the soil on 26 August 2011, 7 November 2011, 18 January 2012, 27 February 2012 and stored at -80°C until they were analysed for ABA. Seeds harvested on 27 May 2011 were also used for the analysis. Before stating the experiments, all seeds were lyophilized and weighed.

Extraction and purification was conducted according to Agar et al. (2006) with a few modifications. Frozen sample was powdered in liquid nitrogen. Then 80% methanol containing 1 mM butylated hydroxytoluene as an antioxidant was added to the fine powder and stored at 4°C for 36 h in the dark. The supernatant was collected by centrifugation at 15,000 rpm for 15 min at 4°C. The precipitate was re-extracted once more in the same way.

Each supernatant was well mixed with 0.2 g polyvinylpyrrolidone (PVPP, Sigma Chemical Co. UK) for 30 min and collected through centrifugation. Then, the supernatants were put in flask (25 cm<sup>3</sup>) and were evaporated off the methanol at 35°C under reduced pressure with a rotary evaporator. The extracts were dissolved in 5% MeOH and filtered with Sep-Pak C18 cartridges (Waters, Hichrom Ltd. UK). The solvent passed through Sep-Pak was discarded. The residue in Sep-Pak cartridges was eluted by 80% MeOH. The filtrates were re-filtered with Sep-Pak C18 cartridges. The hormones were eluted with 80% methanol and collected in vials.

Ten µL of each sample were analyzed with HPLC-MS/MS system in NICEM. A Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap instrument (Thermo Scientific, USA) equipped with Dionex Ultomate 3000 RSLCnano HPLC system was used. Mass spectrometric analyses were performed using a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap instrument mass spectrometer, with ESI interface. Ionization of analytes was carried out using electrospray ionization (ESI). The capillary temperature was maintained at 320°C, the ion source voltage was set at 3.5 kV and the sheath and Aux gas was set at 30 and 5 units. The capillary voltage was set at 3.5kV. The average scan time was 0.01 min while the average time to change polarity was 0.02 min. The HCD energy was generally chosen in order to maintain about 20% abundance of the precursor ion.

## RESULTS

### Effect of exogenous GA and ABA on embryo growth

All embryos treated with distilled water (GA 0) were in the smallest size class (<1.10 mm) (Fig. III-1). When seeds treated with 1,000 mg·L<sup>-1</sup> GA<sub>3</sub>, 38% of the embryos were in the largest (>3.51 mm) size class and 28% of embryos were in the smallest size class. However, length of embryos decreased with increasing ABA concentration. In 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> + 1,000 mg·L<sup>-1</sup> ABA, 86% of embryos were in the smallest size class.

### Effect of GA and fluridone on germination

Seeds treated with distilled water failed to germinate during incubation for 25 weeks or longer (Data not shown). Seeds treated with 1,000 mg·L<sup>-1</sup> of GA<sub>3</sub> germinated to 21% at 11 weeks (Fig. III-2). However, seeds treated with 10 and 100 mg·L<sup>-1</sup> of fluridone germinated to 16 and 50%, respectively, at 11 weeks and to 76 and 87%, respectively, at 22 weeks. When 1000 mg·L<sup>-1</sup> GA<sub>3</sub> + 10 mg·L<sup>-1</sup> fluridone were applied together, 40% of seeds germinated at 11 weeks. However, no germination occurred in 500 mg·L<sup>-1</sup> of fluridone or 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> + 500 mg·L<sup>-1</sup> fluridone.

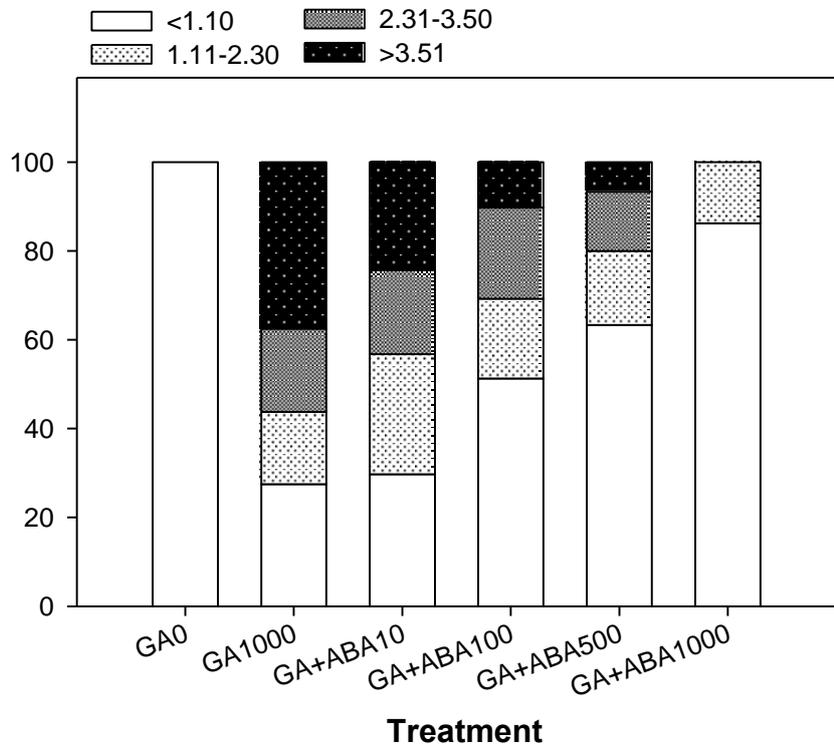


Fig. III-1. Changes in size-class distribution of embryos in 0-month-old (freshly matured) seeds of *Jeffersonia dubia* in water (GA0), in 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> solution, and in a 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> solution combined with either 10, 100, 500, or 1,000 mg·L<sup>-1</sup> ABA.

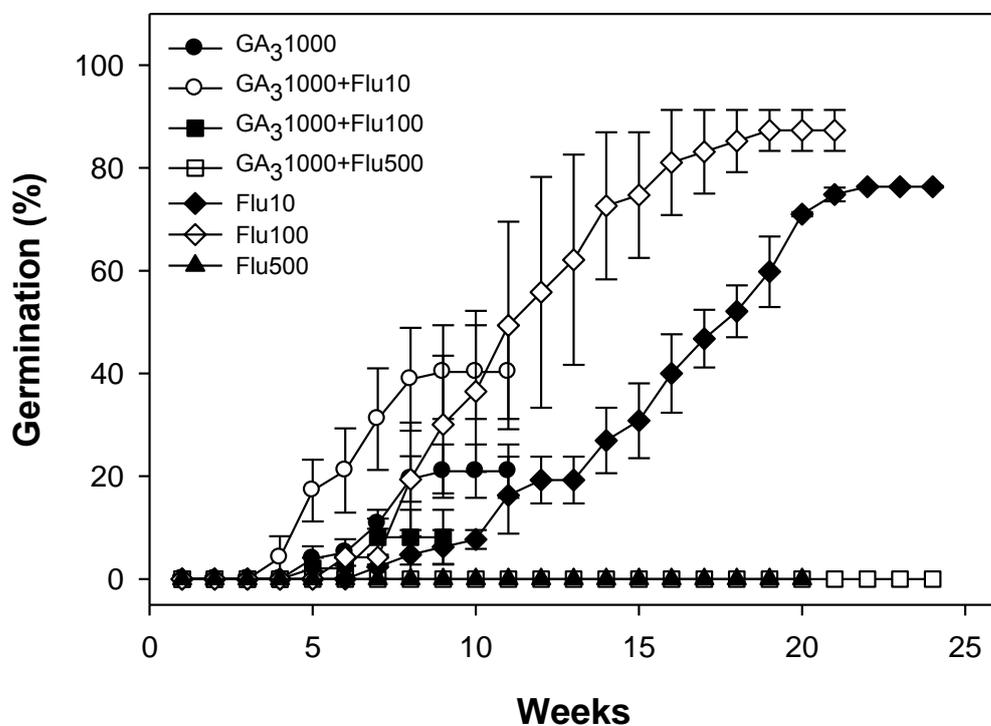


Fig. III-2. Germination in 0-month-old (freshly matured) seeds of *Jeffersonia dubia* treated with various treatments. Dormant seeds were soaked for 24 h in 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> solution, in a 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> solution combined with either 10, 100, or 500 mg·L<sup>-1</sup> of fluridone and in 10, 100, or 500 mg·L<sup>-1</sup> fluridone solutions and then transferred to 20/10°C (day/night). Bars are ± SEs.

### **Effect of ABA and paclobutrazol on germination during cold stratification**

Although seeds incubated at 20/10°C without cold stratification failed to germinate, seeds subjected to the low temperature for 12 weeks in distilled water germinated to 72% for 8 weeks after incubation at 20/10°C (Fig. III-3). Only 4% of seeds treated with 100 mg·L<sup>-1</sup> paclobutrazol germinated though seeds were treated low temperature of 5°C for 12 weeks. When seeds were treated with 100 mg·L<sup>-1</sup> ABA, 50% of seeds germinated.

### **Effect of GA<sub>3</sub> and fluridone on germination in warm stratified seeds**

Germination did not occur in seeds treated with distilled water (GA 0) (Fig. III-4). However, higher concentration of 10, 100, and 1,000 mg·L<sup>-1</sup> allowed 4, 43, and 72% germination, respectively. In presence of 100 mg·L<sup>-1</sup> fluridone the seeds germinated to 100%, while in 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> + 100 mg·L<sup>-1</sup> fluridone the seeds germinated to 84%.

### **Endogenous hormones analyses**

The endogenous ABA was detected in the seeds of *J. dubia* and ABA contents differed among the seasons (Fig. III-5). The amounts of ABA was 268 ng·g<sup>-1</sup> in fresh seeds, 157 ng·g<sup>-1</sup> on 26 August 2011, 32.3 ng·g<sup>-1</sup> on 7 November 2011, 96 ng·g<sup>-1</sup> on 18 January 2012, and 15 ng·g<sup>-1</sup> in germinated seeds on 27 February 2012.

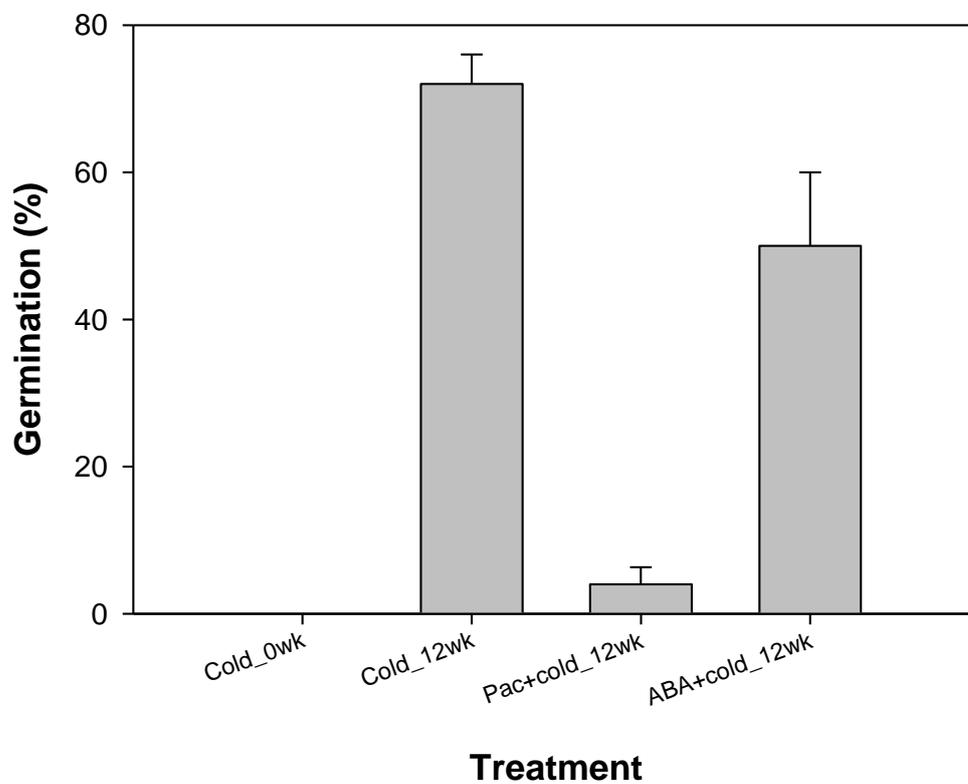


Fig. III-3. Germination in warm-stratified seeds of *Jeffersonia dubia* treated with various treatments. Seeds exhumed from field soil at 10 November 2010 were cold-stratified under 5°C for 0 (Cold\_0wk) or 12 weeks (Cold\_12wk). Seeds were soaked for 24 h in 100 mg·L<sup>-1</sup> paclobutrazol solution (Pac+cold\_12wk) or in 100 mg·L<sup>-1</sup> ABA solution (ABA+cold\_12wk) and then cold-stratified under 5°C for 12 weeks. All treated seeds were incubated at 20/10°C. Bars are ± SEs.

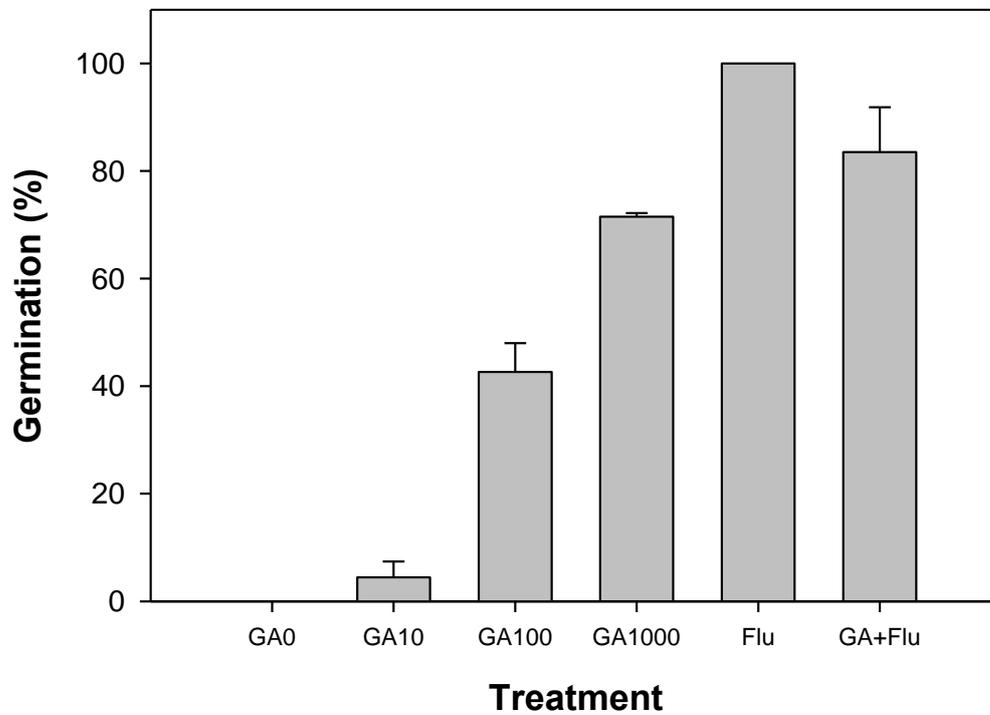


Fig. III-4. Germination in warm-stratified seeds of *Jeffersonia dubia* treated with various treatments. Seeds exhumed from field soil at 10 November 2010 were soaked for 24 h in 0, 10, 100, or 1,000 mg·L<sup>-1</sup> GA<sub>3</sub> solutions. Seeds were soaked for 24 h in a 100 mg·L<sup>-1</sup> fluridone solution combined with either 0 (FLU) or 1,000 mg·L<sup>-1</sup> (GA+FLU) GA<sub>3</sub> solutions. All treated seeds were incubated at 20/10°C. Bars are ± SEs.

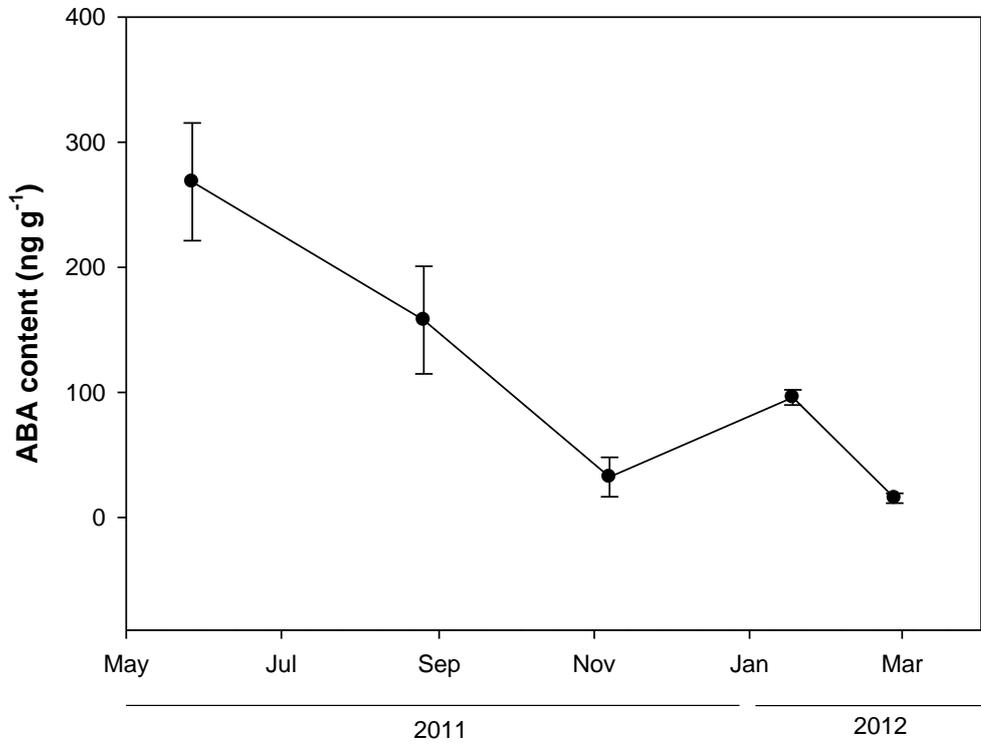


Fig. III-5. The contents of endogenous ABA of the *Jeffersonia dubia* seeds during dormancy breaking.

## DISCUSSION

The seeds of *J. dubia* are deeply dormant and require prolonged warm followed by cold periods of stratification to germination (Rhie et al., 2014). ABA controls germination by inhibiting both the embryo growth potential and the second step of endosperm weakening (da Silva et al., 2004). ABA concentration declines in seeds or in embryo tissues during cold or warm stratification (Pinfiels et al., 1989; Chen et al., 2007). Warm stratification in summer seasons was required for the release of dormancy in the small differentiated embryo (Fig. II-2) and was associated with decline in ABA concentration (Fig. III-5). Studies on woody plants indicated that the GA content of seeds increases during cold stratification. For example, cold stratification induced an increase in GA<sub>3</sub> and in GA<sub>7</sub> in peach seeds (Mathur et al., 1971) and in GA<sub>1</sub> in *Corylus avellana* embryos (Williams et al., 1974). GA<sub>4</sub> contents of embryos from cold stratified seeds were up to three times that of embryos from fresh or warm stratified seeds, and this coincided with a high germination percentage of cold stratified seeds (Chen et al., 2007). Subsequent cold stratification was required for germination of *J. dubia* (Fig. II-2) and might be associated with an increase in GAs concentration.

Exogenous application with 1,000 mg·L<sup>-1</sup> of GA<sub>3</sub> could directly stimulate embryo growth within 4 weeks (Fig. III-1). GA<sub>3</sub> is effective in substituting for warm stratification in seeds with deep simple MPD (Baskin and Baskin, 1998). However, when given in combination with ABA, ABA strongly reverses the dormancy-breaking effect of GA<sub>3</sub> and keeps the embryo small (Fig. III-1). It showed that ABA might antagonize GA responses during warm stratification. In *Panax quinquefolium*, the effectiveness of embryo growth was reduced by combination treatment of GA and ABA compared to treatment of single GA (Ren et al., 1996). We observed that the growth of embryos was suppressed at

higher ABA concentrations. It means that relative amount of GA and ABA controlled the first part of PD.

The second part of PD was broken by prolonged low temperature (Fig. III-3). When seeds were treated with GA, seeds germinate to 72%, indicating that GA could substitute cold stratification (Fig. III-4). Exogenous application of GA<sub>3</sub> or of GA<sub>4+7</sub> has been reported to be effective in breaking dormancy and in substituting for a cold stratification requirement in many seeds (Powell, 1987; Chien et al., 1998). Furthermore, the proportion of GA might influence the break of the second part of PD (Fig. III-4). ABA was also involved with the second part of PD because fluridone or GA + fluridone treatment increased germination. The carotenoid biosynthesis inhibitors, norfluzon and fluridone, have been used in experiments to inhibit ABA biosynthesis (e.g. Grappin et al., 2000; Ali-Rachedi et al., 2004; Chae et al., 2004; da Silva et al., 2004). Grappin et al. (2000) added GA or fluridone to the seeds, both of which prevented ABA biosynthesis and accelerated the rate of germination. ABA treatment inhibits, and fluridone treatment accelerates, radicle protrusion of coffee seeds (da Silva et al., 2004).

During cold stratification, break of dormancy is mediated, at least in part, by promoting GA biosynthesis via enhanced expression of *AtGA3ox* (Yamaguchi and Kamiya 2002; Oh et al., 2004) and by promoting ABA catabolism via activity of the flowering gene *FLC* (*FLOWERING LOCUS C*; Chiang et al., 2009). Germination decreased to <5% when cold stratification was treated along with a GA synthesis inhibitor (Fig. III-3). It means that GA might be essential for the break of the second of PD. There is other evidence that GA is absolute requirement for germination. GA-deficient mutants of tomato (*gib1*) and *Arabidopsis* (*gal*) require exogenous GA to complete germination (Koornneef and Van der Veen, 1980; Groot and Karssen, 1987). On the other hand, ABA treatments decreased

the germination of cold stratified seeds (Fig. III-3). ABA negated the germination-promoting effects (Kucera et al., 2005). ABA inhibits embryo growth potential and endosperm cap weakening during coffee seed germination (da Silva et al., 2004). Overexpression of genes for ABA biosynthesis can increase seed ABA content and enhance seed dormancy or delay germination (Frey et al., 1999; Thompson et al., 2000; Nambara and Marison-Poll, 2003). In seeds with deep simple MPD, both GA and ABA affects the second part of PD. Thus, the second part of PD might be also affected by both GA and ABA.

In seeds with deep simple MPD, GA<sub>3</sub> was not very effective in enhancing germination while GA<sub>3</sub> could promote embryo growth of *J. dubia* (Fig. I-6). GA substitutes for warm stratification but not for cold stratification. Most of seeds exposed to GA<sub>3</sub> decrease in viability and died (Data not shown). We assumed that GA treatment temporarily increased GA/ABA ratio and enhanced embryo growth. However, ABA synthesis and GA degradation might continue to operate in seeds because they had not been taken warm or cold stratification. Eventually, it forced to decrease GA/ABA ratio and its effect leads to disintegration of the seed. However, when seeds were treated with fluridone, up to 70% of seeds germinated and maintained a high viability of seeds (Fig. III-2). It means that ABA might be act as a dominant-negative inhibitor of the entire dormant period. Fluridone could maintain a high GA/ABA ratio. In the present study, we have shown that GA/ABA balance play a role in the regulation of seed dormancy and germination in *J. dubia*.

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## CONCLUSION

Seeds of *J. dubia* have dormancy and require about 10–11 months from seed dispersal to germination in nature. They had underdeveloped embryos at the time of seed dispersal, indicating that they have morphological dormancy (MD). Although seeds were incubated at various temperature regimes (25/15, 20/10, 15/6 or 5°C), none of seeds germinated in several months, indicating that seeds have morphophysiological dormancy (MPD) as well as MD. Among seeds with MPD, if embryos grow at warm temperatures, seeds have simple type dormancy. Embryos of *J. dubia* grew at 25/15°C (day/night) or constant 25°C for 8 weeks or more. Warm stratified seeds, whose embryos had been grown fully, had to be cold stratified for 8 weeks or more for germination. Thus, for germination of *J. dubia* seeds, warm followed by cold stratification treatment is needed. However, if seeds were incubated under low temperature regimes first, embryos did not grow until they were moved to 25/15°C. GA<sub>3</sub> increased the rate of embryo growth in *J. dubia*, indicating that GA<sub>3</sub> could substitute for warm stratification for embryo growth. However, few seeds treated with GA<sub>3</sub> germinated at 20°C. Therefore, GA<sub>3</sub> did not substitute for the low temperature requirement for dormancy breaking, suggesting that seeds have deep simple MPD. Seeds could germinate within 3 months with 1,000 mg·L<sup>-1</sup> GA treatment accompanied by incubation at 15/6°C, where 15°C might act as the moderate temperature for embryo growth after GA treatment whereas 6°C as the subsequent low temperature condition required for completion of radicle protrusion.

In anatomical observation, embryos in seeds of *J. dubia* were embedded in endosperm. Main components of endosperm cell wall were considered as mannan-based polymers because hydrates of cell wall were mainly large quantities of mannose and smaller quantities of galactose. Endo-β-mannanase activity, which was considered the major

enzyme for the degradation of mannan-containing polymers, was not detected during warm stratification (June-August). The increase of enzyme activity in the endosperm coincided with the increase in embryo length within the seed under moderate temperatures (October-November). Although enzyme activity decreased in winter season when embryo ceased to grow, it increased again during germination in spring season. In general, the weakening of lateral or micropylar endosperm was needed for reducing the mechanical restraint of the endosperm cells or for providing the carbohydrate needed for embryo growth. It is suggested that in *J. dubia*, mannan polysaccharides play a role as a carbohydrate source to support germination and the growth of the seedling because enzyme activity significantly increased after radicle protrusion. The pattern of endo- $\beta$ -mannanase during dormancy breaking in seeds with deep level of MPD dormancy was first observed in this study.

High levels of ABA accumulated in freshly harvested seeds. However, the ABA content decreased during warm stratification and the embryo began to grow. Exogenous GA and fluridone treatments also promoted embryo growth in seeds of *J. dubia*, but exogenous ABA restrained the growth of embryos. When seeds were exhumed in November, embryo in seeds was fully grown but dormant. Although these seeds required cold stratification, exogenous GA and fluridone could substitute for cold stratification and promote the radicle protrusion. However, ABA or paclobutrazol treatments suppressed seed germination though seeds were subjected to cold stratification for 8 weeks.

In summary, it is concluded that 1) Seeds of *J. dubia* have deep simple MPD that can be broken by a period of warm followed by cold stratification, 2) Warm stratification decreases endogenous ABA content and increases the embryo growth, 3) Relative amounts of exogenous GA and ABA could control the seed dormancy of deep simple

MPD as well as seed dormancy of non-deep PD or non-dormancy, 4) Endosperm weakening is a pre-germination event and is mediated by the induction of endo- $\beta$ -mannanase activity, resulting in a digestion of the endosperm cell walls, and 5) Following treatment of GA<sub>3</sub>, seeds can germinate at 15/6°C within 3 months. These approaches may be useful in germinating seeds of other native Korean species that previously have proven to be difficult or impossible to germinate.

## ABSTRACT IN KOREAN

본 논문은 갯쟁이풀의 종자휴면을 분류하고 종자 내 배유의 구성성분 조사 및 배유 세포벽 분해 효소인 endo- $\beta$ -mannanase의 변화를 분석하였다. 또한 시기별 식물 호르몬 변화 양상과 GA-ABA의 상대적 비율이 종자의 휴면을 유지하고 타파하는데 어떠한 영향을 미치는지 알아보았다. 채종 당시 갯쟁이풀의 종자는 미성숙배를 가지고 있지만 물 흡수는 잘 되었다. 자연상태에서 종자의 배는 9월에 자라기 시작하며 11월에 배생장이 완성된다. 발아는 이듬해 3월에 이루어지며, 바로 이어 유묘가 출현 한다. 기내 실험에서 배의 성장을 유도하기 위해서는 고온(25°C; 25/15°C)에서 8주 이상 배양 해야 하며 이후 배의 생장은 중온(20/10°C; 15/6°C)조건을 필요로 한다. 이렇게 배가 성숙한 종자는 적어도 5°C, 8주 이상의 저온을 받아야 발아를 하게 된다. 지베렐린(GA<sub>3</sub>)처리는 고온을 대체할 수 있지만 저온을 대체하지는 못했다. 이러한 결과를 바탕으로 갯쟁이풀은 deep simple 형태생리휴면(deep simple morphophysiological dormancy)을 가지고 있음을 결론지을 수 있었다. 자연상태에서는 채종에서 발아까지 10-11개월이 소요되지만 1000 mg·L<sup>-1</sup> GA<sub>3</sub>을 처리하고 15/6°C에서 배양했을 때 3개월만에 발아를 유도할 수 있었다.

종자내 배유 세포벽의 구성성분은 대부분 mannose를 주요 성분으로 하고 소량의 glucose와 galactose의 결합체로 이루어진 다당류로 이루어져 있음을 알았다. 배가 자라지 않는 3개월 동안 endo- $\beta$ -mannanase는 전혀 활성을 보이지 않았다. 9월에 배가 자라기 시작할 때 endo- $\beta$ -mannanase 활성이 배 부근에서 나타나기 시작했으며 이때 배 주변의 lateral 배유 부근에서 세포벽이 붕괴되는 것을 발견하였다. 반면 micropylar 배유에서는 세포벽이 침식이 되지 않음을 관찰하였다. endo- $\beta$ -mannanase의 활성은 배의 성장 속도와 일치하여 나타났으며 배 생장이 멈춘 2-3개월의 겨울 기간 동안 효소 활성은 낮아지게 되었다. 하지만 종자가 발아를 하며 효소활성은 다시 높아지게 되었다. 이러한 계절에 따른 endo- $\beta$ -mannanase의 변화는 deep simple 형태생리휴면에서 나타나는 독특한 현상으로 여겨지며 지금까지 endo- $\beta$ -mannanase의 연구가 주로 이루어진 생리적 휴면 또는 휴면이 없는 종자에서의 효소 발생 양상과는 다른 양상이었다. 또한 외생 GA<sub>3</sub>와 ABA합성 저해제인 fluridone을 처리하였을 때 깻깻이풀의 배의 생장이 촉진되었다. 하지만 외생 ABA를 처리했을 때 배 생장이 저해 받았다. 11월에 굴취한 깻깻이풀 종자는 배가 자랐고 발아를 위해서는 저온을 필요로 한다. 이 종자에 GA나 fluridone을 처리했을 때

발아가 촉진되었으며 반면 ABA나 GA생합성 저해제인 paclobutrazol을 처리했을 때 저온처리를 8주간 해도 발아가 억제되었다. 이러한 결과를 통해 GA와 ABA의 균형에 의해 deep simple 형태생리휴면이 조절되고 있다는 것을 유추할 수 있었다.