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Research article

Effects of moist cold stratification on germination, plant growth regulators, metabolites and embryo ultrastructure in seeds of *Acer morrisonense* (Sapindaceae)

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ABSTRACT

Breaking of seed dormancy by moist cold stratification involves complex interactions in cells. To assess the effect of moist cold stratification on dormancy break in seeds of *Acer morrisonense*, we monitored percentages and rates of germination and changes in plant growth regulators, sugars, amino acids and embryo ultrastructure after various periods of cold stratification. Fresh seeds incubated at 25/15 °C for 24 weeks germinated to 61%, while those cold stratified at 5 °C for 12 weeks germinated to 87% in 1 week. Neither exogenous GA_3 nor GA_4 pretreatment significantly increased final seed germination percentage. Total ABA content of seeds cold stratified for 12 weeks was reduced about 3.3-fold, to a concentration similar to that in germinated seeds (radicle emergence). Endogenous GA_3 and GA_7 were detected in 8-week and 12-week cold stratified seeds but not in fresh seeds. Numerous protein and lipid bodies were present in the plumule, first true leaves and cotyledons of fresh seeds. Protein and lipid bodies decreased greatly during cold stratification, and concentrations of total soluble sugars and amino acids increased. The major non-polar sugars in fresh seeds were sucrose and fructose, but sucrose increased and fructose decreased significantly during cold stratification. The major free amino acids were proline and tryptophan in fresh seeds, and proline increased and tryptophan decreased during cold stratification. Thus, as dormancy break occurs during cold stratification seeds of *A. morrisonense* undergo changes in plant growth regulators, proteins, lipids, sugars, amino acids and cell ultrastructure.

1. Introduction

Cold stratification is commonly used to break dormancy in water-permeable seeds of many temperate-zone species, especially those whose seeds germinate in the natural habitat in spring. In this dormancy-breaking treatment, seeds are incubated on a moist substrate at low (0–10 °C) temperatures. For many species, 5 °C is optimal for dormancy break (Baskin and Baskin, 2014), but in some species temperatures lower than 5 °C are more effective than 5 °C. For example, 0 °C was more effective than 5 °C in overcoming physiological dormancy after the embryo in seeds of *Aegopodium podagraria* had become fully developed (Phartyal et al., 2009). In some species, cold stratification is not very effective in breaking dormancy unless it is preceded by several weeks of warm (≥15 °C) moist stratification, e.g. *Taxus* species (Chien et al., 1998), *Cephalotaxus* (Yang et al., 2011) and some *Fraxinus* (Bonner and Karrfalt, 2008) and *Viburnum* (Chien et al., 2011) species. As dormancy break occurs via cold stratification, germination percentages and rates increase, and often the temperature range over which seeds will germinate increases (Baskin and Baskin, 2014). Also, many biochemical and structural changes are known to occur in seeds during cold stratification (Bewley et al., 2013).

During dormancy-break, the level of abscisic acid (ABA) is high in freshly matured seeds and decreases with warm or cold stratification, and levels of GA_1, GA_3, GA_4 or GA_7 (gibberellins) increase after cold stratification (Chen et al., 2008, 2010; Lewak, 2011). The

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effects of cold stratification on enzymatic activities and reserves in seeds of woody plants have been investigated. Cold stratification (1) broke embryo dormancy of *Malus domestica* seeds via catabolism of lipids, sugars and proteins by hydrolytic or proteolytic enzymes (Lewak, 2011); (2) promoted germination of *Juglans regia* seeds by activating hydrolases that catabolized proteins and thus increased the quantity of amino acids (Einali and Sadeghipour, 2007); and (3) broke dormancy in seeds of *Corylus avellana* and increased starch content, probably as a result of gluconeogenesis from products of reserve lipid hydrolysis (Li and Ross, 1990).

Ultrastructural studies of embryo cells have revealed that there is gradual mobilization of lipid and protein bodies in cold stratified seeds. Cold stratification of *M. domestica* seeds for 90 days not only broke dormancy but caused degradation of protein bodies and lipids, and the degraded lipids supposedly were converted into starch (Dawidowicz-Grzegorzewska, 1989). Warm stratification of yew (*Taxus mairei*) seeds for 6 months decreased the number of lipid and protein bodies and increased the number of mitochondria, plastids, dictyosomes, vacuoles and microbodies in the shoot apical meristem, and then cold stratification for 3 months initiated cell division and finally germination (Chien et al., 1998). Cold stratification of aged *Picea mariana* seeds resulted in repair of ultrastructural damage in the seeds and increased germination percentages (Wang and Berjak, 2000).

Although detailed biochemical and structural studies have been conducted on seeds of various species during cold stratification, our knowledge is deficient in what happens during cold stratification of seeds of some of the major genera of trees. One such genus is *Acer*. This genus consists of about 148 species of deciduous (rarely evergreen) trees and shrubs distributed in the northern hemisphere, including Europe, North America and Asia, which is the geographic center of the genus (Zasada and Strong, 2008). There are about 140 species in China, including six species in Taiwan (Woody Flora of China, 2004). Many species of *Acer* have ornamental value and thus are widely used for landscaping. Fruits (samaras) of most *Acer* species are dormant and require cold stratification or sometimes warm plus cold stratification for dormancy to be broken (Baskin and Baskin, 2014). The length of the cold stratification period required to break dormancy in seeds of *Acer* ranges from c. 45 to 90 up to 180 days, depending on the species (Baskin and Baskin, 2014).

Pinfield and Gwarazimba (1990) found that the ABA content of the embryo in fresh *Acer* seeds was high but decreased with warm and/or cold stratification. The decrease in ABA content, probably in conjunction with weakening of the testa and pericarp, promoted rapid seed germination. The purpose of our research was (1) to determine the effect of cold stratification and exogenous GA_3 and GA_4 on germination, and (2) monitor changes in endogenous levels of ABA, GA, metabolites and embryo ultrastructure during cold stratification of seeds of *Acer morrisonense*. This species is endemic to Taiwan, and it is a dominant tree in forests at elevations of 1800m–2500 m (Li and Lo, 1993; pers. observ.).

2. Materials and methods

2.1. Fruit collection

Freshly matured samaras of *A. morrisonense* were collected from Tsuifeng at an elevation of 2400 m, Nanto County, Central Taiwan (24°06′20″N, 121°11′13″E) during early November 2011 and late December 2014. All studies, except the experiment on effects of exogenous GA_3 and GA_4 on germination were conducted using seeds collected in 2011. The GA experiment was done with seeds collected in 2014. After air-drying at room temperatures (21–23 °C) for 1 week, the wing was removed by hand from each fruit, which were stored for 1 week at 5 °C before use. De-winged fruits (hereafter seeds) were used in all studies. Three hundred dry seeds were frozen in liquid nitrogen and stored at −80 °C to await analyses. Seeds (n = 50) were 5.13 ± 0.52 mm long, 3.51 ± 0.23 mm wide and 2.39 ± 0.42 mm thick, and there were 26,800 seeds per liter and 77,750 seeds per kg. Moisture content of fresh seeds was 15.4 ± 0.3% as determined by oven drying for 17 h at 103 °C (International Seed Testing Association, 2007).

2.2. Effect of temperature and cold stratification on germination

To determine the temperature requirements for germination, fresh seeds were mixed with moist sphagnum moss (cut into small pieces) at a 75–80% moisture content in sealed polyethylene (PE) bags (0.04 mm thick). Seeds were incubated for 36 weeks at 30/20, 30/15, 25/15, 20/10, 15/5 °C and 25 °C. At the alternating temperature regimes, the high and low temperature was given for 12 h each day. Seeds were exposed to light 12 h each day and for those incubated at the alternating temperature regimes during the high-temperature phase of the daily cycle (hereafter light). The light source was white fluorescent tubes, and photon irradiance (400–700 nm) was about 60–80 μmol m^{-2} s^{-1}. The moist sphagnum provided a good germination medium and prevented the spread of mold because it contains the fungus *Trichoderma* and actinomycetes that are antagonistic to microorganisms (Wang et al., 1998). Due to the coarseness of the sphagnum moss, most seeds received some light, but at any given point in time a few of them may have been in darkness. However, at weekly intervals the contents of each bag were poured onto a table to facilitate examination of seeds for germination. After germination was monitored, nongerminated seeds and sphagnum moss were returned to the bag, resulting in a re-shuffling of seeds with regard to their position in/on the sphagnum moss and thus the light they received. Consequently, all seeds were in light part (or all) of the time in the incubator. Each treatment consisted of three replications of 50 seeds each. At 1-week intervals, seeds with a radicle ≥2 mm in length were recorded as germinated and removed from the bag. Results are expressed as mean (±1 SE) germination percentage or as germination speed, i.e. time to reach 50% germination of viable seeds (t_{50}) (Bewley et al., 2013).

To determine the response of seeds to cold stratification, fresh seeds were mixed with moist sphagnum moss (as described above) and placed in darkness for 0 (control), 4, 8, 12 and 16 weeks. After each treatment, seeds were incubated for 12 weeks in light at 25/15 °C as described above. The control was fresh seeds incubated at 25/15 °C throughout the experiment. Three hundred seeds each cold-stratified at 5 °C for 4, 8 and 12 weeks and 200 seeds with radicles emerged were immediately frozen in liquid nitrogen and stored at −80 °C until further analyses could be performed.

2.3. Effect of exogenous GA_3 and GA_4 on germination

To promote infiltration of GA_3 or of GA_4 solutions through the pericarp and seed coat and into the embryo, seeds collected in December 2014 were placed in 0, 25, 250 and 2500 μM solutions of GA_3 (potassium salt, 95% purity, Sigma, St Louis, Missouri, USA) or GA_4 (90% purity, from Professor Lewis N. Mander, Australian National University), which were then immediately placed in a vacuum container at a pressure of 30 cmHg (0.4 atm) for 16 h. Vacuum infiltration previously has been used in testing the effects of GA_3 and of GA_4 on seed germination (Loveys and Jusaitis, 1994; Chen et al., 2007). The treated seeds were mixed with moist sphagnum moss and incubated at 25/15 °C for 56 days. Also, seeds treated with 2500 μM GA_4 were incubated at 25/15 °C for 16 days and then

dissected and photographed.

2.4. Microscopic observations on seeds after 0 and 12 weeks cold stratification

Embryos were excised from freshly matured seeds and from those cold-stratified for 12 weeks. They were fixed with 2.5% glutaraldehyde (in 1.0% sodium phosphate buffer) and then with 1.0% osmium tetraoxide (in 1.0% sodium phosphate buffer) and subsequently dehydrated in an ethanol series and transferred into pure acetone for critical point dehydration (Hitachi Critical Point Dryer, HCP-2, Japan). One of the cotyledons was removed from each embryo to reveal the apical meristem of the plumule or the first true leaves, and then the embryos were coated with IB-2 Ion Coater (Eiko Engineering Co., Japan). The plumular apical meristem, first true leaves and cotyledons were examined with a scanning electron microscope (SEM) (FEI Inspect S, FEI Company, USA) and photographed. For transmission electron microscopy (TEM), embryos were fixed as described above, dehydrated in an acetone series and embedded in Spurr's resin (Spurr, 1969). Ultra-thin sections were made by ultramicrotome (Ultracut E), stained with 5% uranyl acetate aqueous solution and observed with a Hitachi H-7650 TEM (Hitachi High-Tech, Tokyo, Japan).

2.5. Analyses for plant growth regulators in fresh, cold-stratified and radicle-emerged seeds

The complete methods for isolation and analyses of ABA, GA, total soluble sugars, starch, non-polar sugars and amino acids can be found in Appendix A.

Endogenous levels of ABA, GA_1, GA_3, GA_4, GA_7 and GA_{20} were analyzed by gas chromatography–mass spectrometry-selected ion monitoring (GC–MS-SIM). Fresh seeds and those cold-stratified for 4, 8 and 12 weeks as well as radicle-emerged seeds were used in the analyses; there were three replications of each sample (about 200 mg dry sample). The methods for extracting and quantifying ABA, GA_1, GA_3, GA_4, GA_7 and GA_{20} in the tissues have been described by Nakayama et al. (2001) and Chen et al. (2008).

2.6. Analysis for total soluble sugars and starch

Total soluble sugars and starch in fresh seeds, seeds cold-stratified for 4, 8 and 12 weeks and radicle-emerged seeds were extracted and then quantified using a spectrophotometer at a wavelength of 490 nm, following the methods for extraction and preparation of test solutions and standards of Yoshida et al. (1976). There were three replications of each sample.

2.7. Analysis for non-polar soluble sugars

Gas chromatography–mass spectrometry was used to identify and quantify the various non-polar sugars in fresh seeds, seeds cold-stratified for 4, 8 and 12 weeks and radicle-emerged seeds. The methods for this analysis follow those of Chien et al. (1996) and Fiehn et al. (2000). There were three replications of each sample.

2.8. Analysis for amino acids

Gas chromatography–mass spectrometry was used to identify and quantify the various amino acids in fresh seeds, seeds cold-stratified for 4 and 8 weeks and radicle-emerged seeds; a minimum five replications of each sample was used. The methods for this analysis followed those of the Metabolomics Fiehn Lab, UC Davis, http://fiehnlab.ucdavis.edu/publications/Arabidopsis Protocols.

2.9. Statistical analyses

Germination data were converted to percentages, and means and standard errors were calculated. Statistical analyses, performed at the 5% level of significance, of the effect of exogenous GA_3 and GA_4 on germination and concentrations of endogenous ABA, GA_1, GA_3, GA_4, GA_7, GA_{20}, non-polar sugars and amino acids in seeds after various periods of cold stratification were carried out using the GLM procedure of SAS, and means were compared by Least Significant Difference (SAS Institute Inc., Cary, North Carolina, USA). Germination percentage data of seeds were arcsine square-root transformed before analysis, but only nontransformed data are shown in tables and figures.

3. Results

3.1. Effect of temperature and cold stratification on germination

Freshly harvested seeds germinated to <4% during incubation in light at 30/20, 30/15, 25/15, 20/10, 15/5 and 25 °C for 4 weeks, but after 36 weeks incubation seeds had germinated to 42, 73, 61, 59, 83 and 13%, respectively (Fig. 1a). By week 24, seeds incubated at 15/5 °C had reached a germination plateau of 83%, whereas those at the other temperatures had germinated to lower percentages and had not reached a plateau. Thus, the optimum temperature for germination of *A. morrisonense* seeds was 15/5 °C.

Moist cold stratification at 5 °C significantly increased seed

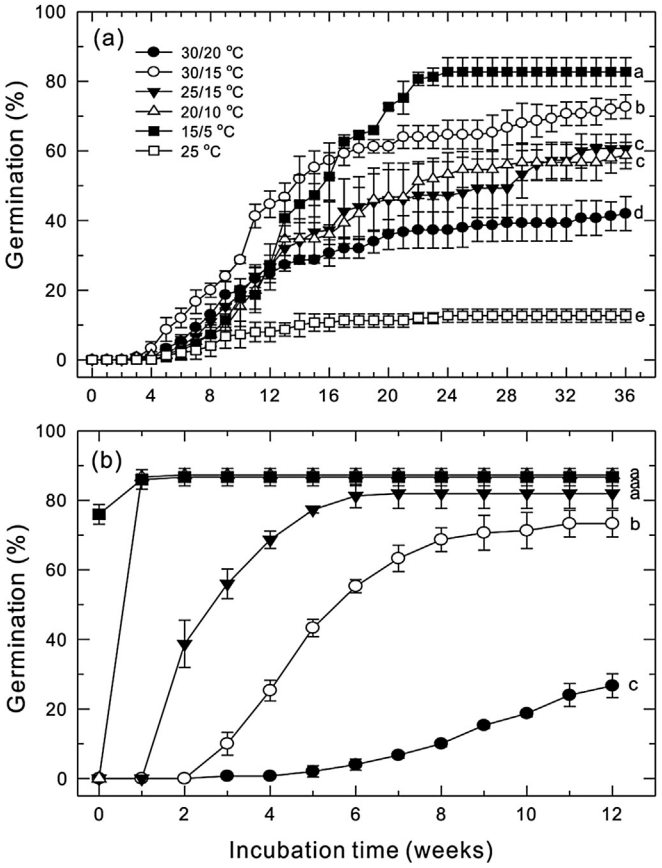

Fig. 1. Cumulative germination percentages of fresh *Acer morrisonense* seeds incubated at six temperature regimes (a), and of seeds stratified at 5 °C in dark for 0 (control, ●), 4 (○), 8 (▼), 12 (△) and 16 (■) weeks and then incubated in light at 25/15 °C after cold stratification (b). Error bars are ±1 SE. Final percentages of germination after 36 weeks followed by different letters differ significantly (LSD, $\alpha = 0.05$).

Table 1
Time (days) to 50% germination (t_{50}) of fresh seeds of *Acer morrisonense* incubated at six temperatures and of seeds cold stratified for 4, 8, 12 and 16 weeks and then incubated at 25/15 °C. Means ($n = 3$) in a column with different lowercase letters differ significantly (LSD, $\alpha = 0.05$). For 25/15 °C, means ($n = 3$) in a row with different lowercase letters are significantly different.

Incubation temperature (°C)	t_{50} of fresh seeds	t_{50} of seeds cold stratified for 4 weeks	t_{50} of seeds cold stratified for 8 weeks	t_{50} of seeds cold stratified for 12 weeks	t_{50} of seeds cold stratified for 16 weeks
30/20	>252	–	–	–	–
30/15	94.3 ± 7.9^{b}	–	–	–	–
25/15	153.4 ± 40.5^{a}	38.8 ± 0.8^{b}	18.2 ± 2.3^{cd}	4.0 ± 0.5^{cd}	0^{d}
20/10	151.1 ± 20.8^{a}	–	–	–	–
15/5	106.1 ± 9.5^{b}	–	–	–	–
25	>252	–	–	–	–

–, experiment not performed; 252 days means seeds did not germinate to 50% in 36 weeks of incubation; 0 days means seeds germinated to ≥50% at 5 °C cold stratification prior to incubation.

Table 2
Effect of GA_3 and GA_4 on germination percentages (mean ± SE) of *Acer morrisonense* seeds after 9, 16, 23, 30 and 37 days incubation at 25/15 °C. Seeds collected in December 2014 were soaked in solutions of GA_3 or GA_4 or in ddH_2O in a vacuum chamber at 30 cmHg for 16 h prior to incubation. Means ($n = 3$) in a column followed by a different letter differ significantly. ***, significant at the 0.001 level and NS, nonsignificant.

Treatments	9 d	16 d	23 d	30 d	37 d
Seeds + ddH_2O (control)	0	5.3 ± 2.5^{c}	28.0 ± 5.7^{b}	41.3 ± 10.4^{a}	46.0 ± 8.6^{a}
+25 μM GA_3	0	10.0 ± 1.6^{bc}	32.0 ± 5.9^{ab}	41.3 ± 5.7^{a}	42.7 ± 5.7^{a}
+250 μM GA_3	0	8.0 ± 1.6^{bc}	34.0 ± 3.3^{ab}	38.0 ± 5.7^{a}	39.3 ± 4.7^{a}
+2500 μM GA_3	0	10.7 ± 3.8^{b}	42.7 ± 3.8^{a}	51.3 ± 8.1^{a}	52.0 ± 7.1^{a}
Seeds + 25 μM GA_4	0	13.3 ± 1.9^{ab}	36.7 ± 1.9^{ab}	41.3 ± 0.9^{a}	42.7 ± 0.9^{a}
+250 μM GA_4	0	13.3 ± 3.4^{ab}	37.3 ± 9.4^{ab}	41.3 ± 10.9^{a}	42.7 ± 9.0^{a}
+2500 μM GA_4	1.3 ± 0.9	18.7 ± 3.8^{a}	42.7 ± 8.4^{a}	46.7 ± 9.6^{a}	49.3 ± 9.8^{a}
Significance					
GA (GA_3, GA_4)		0.0008^{***}	0.1163^{NS}	0.9416^{NS}	0.9729^{NS}
Concentration		0.2107^{NS}	0.1578^{NS}	0.2479^{NS}	0.1617^{NS}

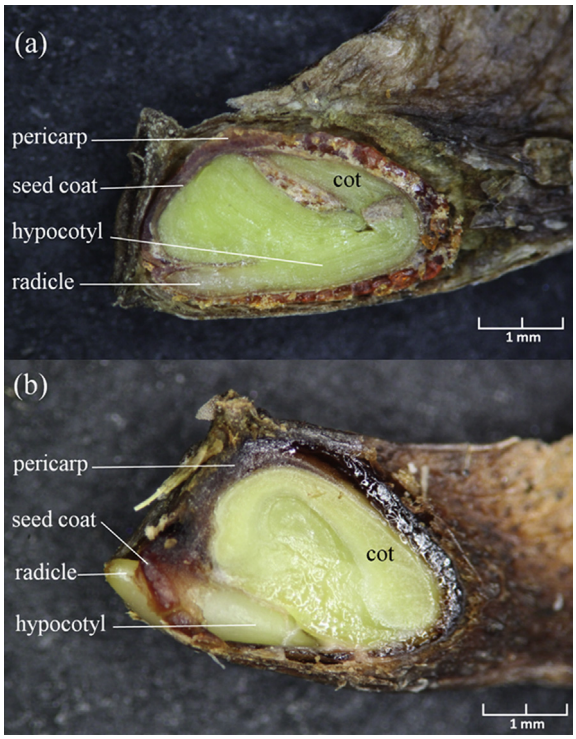

Fig. 2. Longitudinal section of fresh seed (a) and of seed soaked in 2500 μM GA_4 at a vacuum of 30 cmHg for 16 h and then incubated at 25/15 °C for 16 days (b). Radicle emerged and penetrated through seed coat and pericarp rapidly in response to GA. Cot, cotyledons.

germination percentage and speed. For example, seeds cold-stratified for 8 weeks germinated to 82% after 6 weeks incubation at 25/15 °C, while those cold-stratified for 12 weeks germinated to 87% after 1 week at 25/15 °C. After 16 weeks of cold stratification, 76% of the seeds had germinated at 5 °C (Fig. 1b). t_{50} of seeds incubated continuously at the six temperatures ranged from 94.3 days at 30/15 °C to >252 days at 30/20 and 25 °C, and the t_{50} of seeds receiving 4, 8 and 12 weeks cold stratification and tested at 25/15 °C was reduced from 153.4 days for fresh seeds to 4.0 days for seeds cold-stratified for 12 weeks (Table 1).

3.2. Effect of exogenous GA_3 and GA_4 on germination

After 16 days of incubation, seeds treated with 2500 μM GA_3 and with 25, 250 and 2500 μM GA_4 germinated to significantly higher percentages than the control (Table 2). After 23 days of incubation, seeds treated with 2500 μM GA_3 and 2500 μM GA_4 germinated to significantly higher percentages than the control, but after 30 and 37 days there were no significant differences between any of the treatments and the control. Radicle emergence through the seed coat and pericarp occurred within 16 days when seeds were treated with 2500 μM GA_4 and incubated at 25/15 °C (Fig. 2).

3.3. Microscopic observations on seeds after 0 and 12 weeks cold stratification

In freshly matured seeds, two small first true leaves 142.4 ± 16.9 μm in length were observed on the epicotyl plumule (Fig. 3a), and they grew to a length of 430.9 ± 24.5 μm during 12 weeks of cold stratification (Fig. 3b). Many small lipid bodies and some large protein bodies and vacuoles were observed in all cells in the plumule apical meristem of fresh seeds (Fig. 3c). The number of lipid and protein bodies was reduced greatly during cold stratification for 12 weeks, and cell division resulted in formation of new

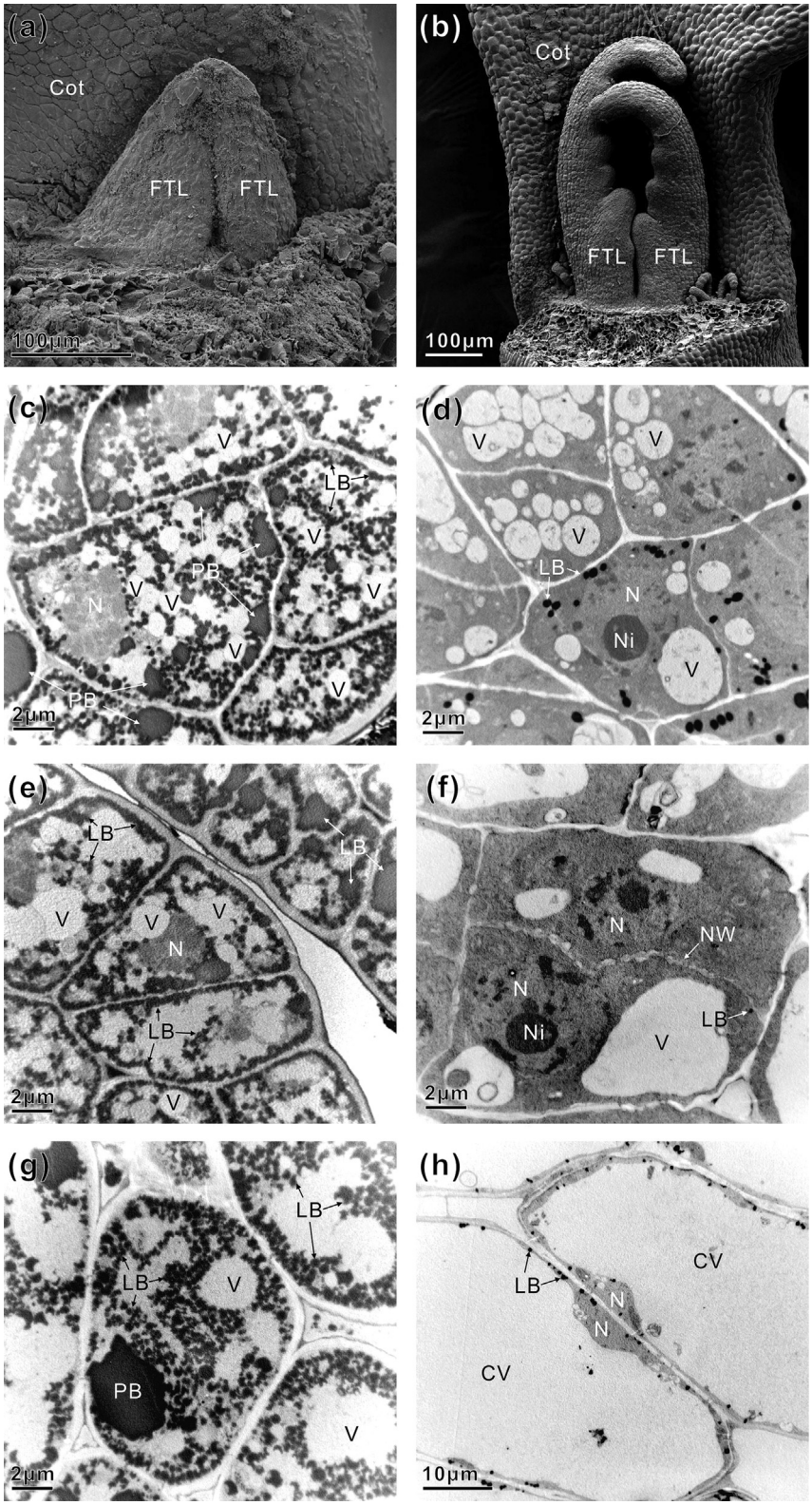

Fig. 3. Electron micrographs showing morphological and anatomical characteristics of freshly matured seeds of *Acer morrisonense* (a, c, e, g) and of seeds cold-stratified for 12 weeks (b, d, f, h). Cotyledons (Cot) and small first true leaves (FTL) on plumule of seed (a, b), and cells of plumular apical meristem (PAM) (c, d), FTL (e, f) and Cot (g, h). In contrast to fresh dormant seeds, the size of FTL of cold-stratified seeds increased 200–300% and the lipid bodies (LB) and protein bodies (PB) decreased in PAM, FTL and Cot. Further, we observed nucleoli (Ni) within nuclei (N) in PAM and FTL, newly formed cell wall (NW) in FTL cells and large central vacuole (CV) in Cot. V, vacuole.

cells (Fig. 3d). Many small lipid bodies and some vacuoles, but no protein bodies, were observed in cells of the first true leaves of fresh seeds (Fig. 3e), and the lipid bodies decreased greatly in number during cold stratification for 12 weeks (Fig. 3f). Cells in the cotyledons of fresh seeds contained many lipid bodies and one large protein body (Fig. 3g), and a large central vacuole was observed

Table 3
Concentration (ng (g DW)$^{-1}$) of endogenous GAs and ABA in fresh, cold-stratified and radicle-emerged seeds of *Acer morrisonense*[a]. Means ($n = 3$) in a row followed by different uppercase letters differ significantly.

	Fresh seeds	4-week cold stratified seeds	8-week cold stratified seeds	12-week cold stratified seeds	Radicle-emerged seeds[b]
ABA	1860.5 ± 295.6^{A}	1291.1 ± 180.5^{B}	934.5 ± 80.8^{C}	566.0 ± 46.2^{D}	543.9 ± 50.7^{D}
GA_1	ND[c]	ND	ND	ND	ND
GA_3	ND	ND	1.9 ± 0.1	0.87 ± 0.10	ND
GA_4	18.2 ± 1.9^{B}	15.5 ± 1.3^{BC}	23.6 ± 1.7^{A}	19.7 ± 0.2^{B}	22.4 ± 1.0^{A}
GA_7	ND	ND	10.5 ± 2.1	10.9 ± 3.5	ND
GA_{20}	ND	ND	ND	1.5 ± 1.1	ND

[a] Wings of fruits were removed prior to seed extraction.
[b] Seeds with emerged radicle were obtained from cold-stratified seeds and then incubated at 25/15 °C.
[c] ND, not detected, but GA internal standard was detected.

Table 4
Total soluble sugar and starch (mg (g DW)$^{-1}$) from fresh, cold-stratified for 4, 8 and 12 weeks and radicle-emerged seeds of *Acer morrisonense*. Means ($n = 3$) in a column followed by different letters differ significantly.

Stage	Total soluble sugar	Total starch
Fresh seeds	18.5 ± 0.8^{b}	16.0 ± 0.9^{a}
seeds cold-stratified for 4 weeks	19.9 ± 0.5^{ab}	16.1 ± 0.4^{a}
Seeds cold-stratified for 8 weeks	20.6 ± 0.4^{ab}	17.7 ± 0.6^{a}
Seeds cold-stratified for 12 weeks	20.9 ± 0.7^{a}	17.3 ± 0.2^{a}
Radicle-emerged seeds	22.0 ± 1.0^{a}	18.0 ± 0.5^{a}

after cold stratification for 12 weeks (Fig. 3h).

3.4. Analysis for plant growth regulators

ABA concentration in fresh seeds was 1860.5 ng (g DW)$^{-1}$, and in seeds cold stratified for 4, 8 and 12 weeks it decreased significantly to 1291.1, 934.5 and 566.0 ng (g DW)$^{-1}$, respectively. ABA content was 543.9 ng (g DW)$^{-1}$ in radicle-emerged seeds. There was no significant difference in ABA content between 12 weeks cold-stratified seeds and radicle-emerged seeds (Table 3). The biologically active GA_3, GA_4 and GA_7 and the immediate precursor GA_{20} of GA_1, but not GA_1, were detected in *A. morrisonense* seeds. GA_4 was detected in fresh, cold stratified and radicle-emerged seeds, GA_3 and GA_7 in seeds cold-stratified for 8 and 12 weeks and GA_{20} in seeds cold stratified for 12 weeks (Table 3).

3.5. Analysis for total soluble sugars and starch

Total concentrations of soluble sugars and starch in fresh seeds were 18.5 and 16.0 mg (g DW)$^{-1}$, respectively. Total soluble sugar concentrations in seeds cold-stratified for 4, 8 and 12 weeks were 19.9, 20.6 and 20.9 mg (g DW)$^{-1}$, respectively, and total starch concentrations were 16.1, 17.7 and 17.3 mg (g DW)$^{-1}$, respectively. Total soluble sugar and starch contents in radicle-emerged seeds were 22.0 and 18.0 mg (g DW)$^{-1}$, respectively (Table 4). There were no significant differences in total starch of fresh, cold-stratified and radicle-emerged seeds, but total soluble sugar increased significantly in 12-week cold stratified and in radicle-emerged seeds (Table 4).

3.6. Analysis for non-polar soluble sugars

Eleven non-polar sugars were detected in fresh seeds of *A. morrisonense*, and the rank order of their abundance was sucrose > fructose > myo-inositol > glucose > raffinose > mannose (Table 5). Sucrose concentration in fresh seeds was 11.8 mg (g DW)$^{-1}$ and it increased to 15.4 mg (g DW)$^{-1}$ during cold stratification for 4 weeks and remained at this level through 12 weeks of cold stratification. However, sucrose decreased to 5.9 mg (g DW)$^{-1}$ in radicle emerged seeds. Fructose decreased about four-fold during cold stratification, and it continued to decrease when the radicle emerged. Myo-inositol, glucose and mannose decreased about 50% during cold stratification; however, they rapidly increased three-to four-fold in radicle-emerged seeds. Raffinose remained at the same level in fresh and cold-stratified seeds but decreased about seven-fold as the radicle emerged. The concentration of melibiose was 44 μg (g DW)$^{-1}$ in fresh seeds, and it was significantly reduced to 23–26 μg (g DW)$^{-1}$ in cold-stratified and radicle-emerged seeds. The concentration of trehalose remained at about the same level, 44 μg (g DW)$^{-1}$, in fresh, cold-stratified and radicle-emerged seeds (Table 5).

3.7. Analysis for amino acids

Eleven amino acids were detected in fresh seeds of *A. morrisonense* (Table 6). The concentration of most of them was low in fresh seeds but increased in cold-stratified and radicle-emerged seeds. Asparagine, glycine and phenylalanine were detected only in radicle-emerged seeds. The concentration of tryptophan in cold-stratified and radicle-emerged seeds decreased to less than one-half of that in fresh seeds.

4. Discussion

Dormancy was broken completely during 24 weeks of incubation at 15/5 °C, but dormancy break was still continuing at 20/10 °C. At these temperature regimes, ngseeds were cold stratified at night when the temperature was at 5 and 10 °C, respectively, indicating that effective cold stratification for *Acer* species is ≤10 °C. Size of the embryo in seeds of *A. morrisonense* did not change before radicle emergence. However, length of the first true leaves on the epicotyl plumule increased 300% during cold stratification for 12 weeks, and newly-formed cells were observed in the plumular apical meristem (Fig. S1, Appendix B) and in the first true leaves (Fig. 3f; Fig. S2, Appendix B), indicating that cold stratification induced cell division and plumule growth.

Dense accumulations of lipid and protein bodies were visible in the plumule apical meristem, first true leaves and cotyledons of fresh seeds (Fig. 3, Fig. S3). However, the number of lipid and protein bodies in fresh seeds was reduced during cold stratification. Presumably, lipids were hydrolyzed to fatty acids and sugars by lipolytic enzymes and storage proteins cleaved to oligopeptides and amino acids by active proteolytic enzymes (Bewley et al., 2013). In seeds of *M. domestica*, proteolytic activity increased markedly during cold stratification, indicating proteolytic solubilization of insoluble reserve proteins, and an increase in quantity of free amino acids paralleled the increase in seed germinability (Lewak et al., 1975; Dawidowicz-Grzegorzewska, 1989). Likewise, acid lipases were most active at low temperature, and enzymes also were activated by GA treatment (Żarska-Maciejewska, 1992). Recently, it was found that protein bodies in olive seeds not only serve as

Table 5
Concentration (μg (g DW)$^{-1}$) of non-polar soluble sugars in fresh, cold-stratified and radicle-emerged seeds of *Acer morrisonense*. Means ($n = 3$) in a row followed by different letters differ significantly.

Sugars	Fresh seeds	4-week cold-stratified seeds	8-week cold-stratified seeds	12-week cold-stratified seeds	Radicle-emerged seeds
Fructose	8712.1 ± 754.3^{a}	2116.3 ± 125.7^{b}	2066.6 ± 43.7^{b}	2504.0 ± 138.9^{b}	1816.9 ± 143.2^{b}
Galactose	110.6 ± 68.5^{a}	57.3 ± 22.0^{a}	63.1 ± 16.6^{a}	78.3 ± 48.6^{a}	68.8 ± 10.5^{a}
Glucose	398.4 ± 44.2^{b}	213.9 ± 29.5^{c}	215.6 ± 18.7^{c}	348.7 ± 34.9^{b}	1283.7 ± 61.4^{a}
Lactose	19.9 ± 1.2^{a}	15.3 ± 2.1^{a}	15.2 ± 0.6^{a}	16.9 ± 2.7^{a}	20.2 ± 7.9^{a}
Maltose	90.4 ± 26.0^{c}	231.8 ± 4.7^{c}	185.7 ± 23.1^{c}	465.8 ± 135.1^{b}	674.1 ± 16.2^{a}
Mannose	271.7 ± 25.9^{b}	143.8 ± 19.4^{c}	147.2 ± 12.3^{c}	229.8 ± 22.0^{b}	854.2 ± 39.6^{a}
Melibiose	44.1 ± 9.2^{a}	23.6 ± 2.8^{b}	23.2 ± 2.0^{b}	26.1 ± 6.7^{b}	15.7 ± 3.0^{b}
Myo-inositol	491.3 ± 13.1^{b}	269.5 ± 8.7^{c}	293.5 ± 5.8^{c}	290.1 ± 14.5^{c}	1213.8 ± 106.2^{a}
Raffinose	357.7 ± 6.5^{a}	355.4 ± 25.6^{a}	391.1 ± 31.4^{a}	342.2 ± 23.2^{a}	49.7 ± 6.8^{b}
Sucrose	11840.8 ± 237.7^{b}	15450.6 ± 792.9^{a}	15907.3 ± 1428.8^{a}	16489.0 ± 1905.6^{a}	5876.9 ± 333.7^{c}
Trehalose	51.3 ± 20.1^{a}	42.3 ± 6.5^{a}	46.0 ± 9.3^{a}	44.2 ± 10.2^{a}	45.8 ± 15.6^{a}

Table 6
Concentration (μg (g DW)$^{-1}$) of amino acids in fresh, cold-stratified and radicle-emerged seeds of *Acer morrisonense*. Means ($n = 3$) in a row followed by different letters differ significantly.

Amino acids	Fresh seeds	4-week cold-stratified seeds	8-week cold-stratified seeds	Radicle-emerged seeds
Alanine	24.9 ± 1.7^{b}	0	0	63.9 ± 1.1^{a}
Asparagine	0	0	0	13.7 ± 4.7
Aspartic acid	10.4 ± 0.2^{c}	10.7 ± 1.6^{c}	19.6 ± 1.7^{b}	34.8 ± 3.2^{a}
Glutamine	0	12.9 ± 3.3^{b}	15.6 ± 5.0^{ab}	18.1 ± 2.6^{a}
Glycine	0	0	0	32.6 ± 1.3
Phenylalanine	0	0	0	33.1 ± 6.3
Proline	77.9 ± 4.8^{c}	108.1 ± 13.2^{b}	121.4 ± 13.0^{b}	157.2 ± 2.5^{a}
Serine	0	13.4 ± 2.0^{c}	50.7 ± 8.9^{b}	319.2 ± 30.7^{a}
Threonine	5.0 ± 1.1^{c}	6.1 ± 2.7^{c}	20.5 ± 1.5^{b}	81.7 ± 9.7^{a}
Tryptophan	33.2 ± 10.0^{a}	32.3 ± 9.1^{a}	15.2 ± 0.8^{b}	14.9 ± 1.3^{b}
Valine	5.4 ± 0.3^{b}	6.9 ± 2.7^{b}	3.8 ± 0.5^{c}	28.6 ± 9.6^{a}

dynamic and multifunctional organelles but that they also are involved in storage lipid mobilization during germination (Jimenez-Lopez and Hernandez-Soriano, 2013; Zienkiewicz et al., 2014). In seeds of *A. morrisonense*, cold stratification increased the content of soluble sugars and most amino acids, and this was followed by enhanced cell division and seed germination.

Enlarged vacuoles were observed in the plumular apical meristem, first true leaves and cotyledons of cold stratified seeds (Fig. 3d, f, h), suggesting that reserves in the lipid and protein bodies were depleted, resulting in the formation of vacuoles and more water in organelles that support metabolism or expansion. Seed germination in *A. morrisonense* is epigeal, and thus the cotyledons are carried above the soil surface, where they expand and become photosynthetic. The large central vacuole (Fig. 3h) in the cotyledon cells that contains nutrients, enzymes and secondary metabolites remain for a while, but it ultimately degenerates.

Control of seed dormancy may depend on the endogenous balance of GA and ABA biosynthesis and catabolism and thus on the ABA:GA ratio rather than amounts of these two plant growth regulators. ABA synthesis and signaling dominate the dormant state and GA synthesis and signaling the transition to germination (Finch-Savage and Leubner-Metzger, 2006). In our study, decrease of the ABA:GA ratio during cold stratification for 12 weeks was correlated with dormancy break. In seeds of *Arabidopsis thaliana*, ABA regulated mobilization of storage lipid in the endosperm (Penfield et al., 2006). Thus, the decrease of ABA content may cause lipid breakdown in cotyledons of *Acer* seed during cold stratification.

Mature seeds of *Acer platanoides* have deep physiological dormancy (Nikolaeva, 1969), and the abundant proteins present in mature seeds when dormancy is established may be involved in energy and carbon metabolism and cellular and antioxidant processes (Staszak and Pawłowski, 2014). Dormancy breaking during cold stratification of *A. platanoides* seeds was accompanied by changes in the proteosome proteins, ABA- and GA-responsive proteins, glycine-rich RNA binding protein, AB13-interacting protein, EF-2 (elongation factor 2) and S-adenosylmethionine synthetase (Pawłowski, 2009). The storage proteins in the plumular apical meristem and cotyledons are subjected to proteolysis, and the amino acids are reutilized to synthesize more proteins (enzymes) during dormancy breaking. Following germination, amino acids from the cotyledons are transported to the growing regions, radicle and first true leaves and used in protein synthesis.

The large embryo of *A. morrisonense* is surrounded by a thin seed coat and thick pericarp (Fig. 2), both of which are water permeable, i.e. 79% increase in mass when seeds were soaked in 2500 μM GA_3 for 16 h (Chen and Chien, unpubl. data). After 7 weeks incubation at 25/15 °C, seeds soaked in 25, 250 and 2500 μM GA_3 and in 25, 250 and 2500 μM GA_4 had germinated to 13.3%–25.3%, but only seeds soaked in 2500 μM GA_3 germinated to a significantly higher percentage than the control. After 35 weeks, seeds soaked in 250 and 2500 μM GA_3 and in 25 and 250 μM GA_4 germinated to a significantly higher percentage than the control (data not shown). However, vacuum infiltration with 2500 μM GA_3 as well as 25, 250 and 2500 μM GA_4 significantly increased germination after only 16 days (Table 2). By 30 days, there were no significant differences between the control and seeds infiltrated with either GA_3 or GA_4. Thus, both GA_3 and GA_4 promoted germination when seeds were soaked or infiltrated, but the response was faster with infiltration.

Sucrose was the most abundant sugar in the seeds of *A. morrisonense*, and its concentration significantly increased after cold stratification for ≥4 weeks (Table 5). Meanwhile, sucrose was catabolized significantly after the radicle emerged, suggesting that the sucrose that accumulated in the cold-stratified seeds was a source of energy for the germinating seeds. Decrease in the concentration of fructose, galactose, glucose, melibiose and myo-inositol during cold stratification may be involved in energy

supply for cell division. Myo-inositol, raffinose and trehalose accumulated in fresh seeds, and these non-reducing sugars play a role in desiccation tolerance of seeds with orthodox storage behavior (Horbowicz and Obendorf, 1994). The concentration of myo-inositol was reduced about 40–45% during cold stratification of *A. morrisonense* seeds, but that of raffinose and of trehalose did not change, indicating that they were not involved in catabolism. Seeds of *A. morrisonense* have orthodox seed storage behavior (Yang and Lin, 1999). The concentration of maltose and of mannose significantly increased in radicle-emerged seeds, and these sugars functioned the same way as glucose in promoting seed germination and seedling growth (Table 5). The total (small) amounts of starch did not differ in fresh, cold-stratified and radicle-emerged seeds, whereas total soluble sugars increased in seeds cold stratified for 4–12 weeks and in radicle-emerged seeds (Table 4). Thus, sugars were mobilized within the seed, and they provided energy for support early seedling growth.

5. General summary/conclusion

The beneficial effects of cold stratification on breaking dormancy and promoting seed germination of many tree species including *Acer* are well recognized. However, we lack a full understanding of the biochemical and cell ultrastructural changes during cold stratification. In our study, 12 weeks of cold stratification were required for dormancy break, during which the ABA/$GA_{3, 4, 7}$ ratio decreased and storage proteins and lipids were hydrolyzed, resulting in changes in sugars and amino acids. Furthermore, cell division and embryo axis growth occurred late in the cold sratification period. Then, radicle emergence soon followed in seeds incubated at a warm temperature and in those kept at 5 °C for a short period following dormancy break. Although we have shown that biochemical and cell ultrastructural changes occur in seeds of *A. morrisonense* during the dormancy breaking cold stratification process, identification of the precise dormancy breaking mechanism requires further study.

Author contributions

SYC and CTC conducted the experimental work and wrote the initial version of the manuscript; SHC, CCT and WYH performed the experiments and data analysis; CCB, JMB revised several drafts of the manuscript; and LLKH and CCT did the microscopic analysis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.06.004.

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