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The Role of Abscisic Acid in Heat Stress-induced Secondary Dormancy in Apple Seeds

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Abstract. **Exposure of stratified apple *(Malus domestics* Borkh. cv. Golden Delicious) seeds to 30C induces secondary dormancy. To determine if an increase in abscisic acid (ABA) content was associated with the loss in germination capacity, stratified seeds (3,- 6, or 9 weeks at 5C) were held at 30C for 0, 3, or 6 days. Stratification at 5C either had no effect or increased ABA content in embryonic axes, cotyledons, and seed coats. Exposure to 30C after stratification either did not affect or decreased ABA content of embryonic axes and seed coats; in contrast, cotyledonary ABA was increased. Seed coats, cotyledons, and embryonic axes stratified for 3, 6, or 9 weeks at 20C contained the same or higher levels of ABA in comparison with nonstratified seeds or seeds stratified at SC. Changes in ABA levels were not consistently correlated with changes in germination capacity during stratification or after exposure to 30C. These data suggest that changes in ABA are not related to changes in dormancy. Chemical names used: abscisic acid (ABA); butylated hydroxy-toluene (BHT); N-(trichloromethyl) thio-4-cyclohexene-1,2-dicarboximide(Captan).**

Since its chemical identification in 1965 (Ohkuma et al., 1965), ABA has become the primary growth inhibitor studied in plants. Pieniazek and Rudnicki (1967) first detected the presence of an ABA-like inhibitor in dormant apple seeds. Attempts to correlate the levels of ABA with the induction of dormancy in seeds have been inconclusive. Rudnicki (1969) reported that inhibitory activity in diffusates from seeds declined with stratification. In contrast, Balboa-Zavala and Dennis (1977) and Subbaiah (1987) observed no relationship between the breaking of primary dormancy and ABA content in apple seeds. Balboa-Zavala and Dennis (1977) measured ABA levels in stratified apple seeds during the induction of secondary dormancy. They found that ABA levels in whole seeds decreased during the period of high-temperature exposure. To clarify the role of ABA in the induction of secondary dormancy, we measured the levels of ABA in the seed coat, cotyledons, and embryonic axes of stratified and nonstratified apple seeds before and after secondary dormancy was induced by exposure to high temperature.

Seed source and methods of stratification. Seeds were removed from Frazier Spur 'Golden Delicious' fruit at harvest, dried, and stored at 5C. Subsequently, seeds were soaked overnight in water, rinsed, placed in 150 × 15-mm plastic petri plates (400 seeds per plate) on filter paper wetted with 0.5% Captan solution, and held at 5 or 20 ± 1C in the dark for 0, 3, 6, or 9 weeks.

Experimental conditions. After stratification, seeds (15 per replication, three replications per treatment) were rinsed, placed in 100 x 15-mm petri plates containing the same solution used for stratification, and held at 30C for 0, 3, or 6 days. Seeds were then dissected into embryonic axes (125; 54 mg), cotyledons (200; 2.1 g) and testa plus endosperm (= seed coat; 100; 1.4 g) and analyzed for ABA content (three replications per treatment). Seeds held at 20C were dissected immediately after 20C exposure.

Evaluation of germination. The results are presented as the percentage germination summed over 10 days (Sum 10; 1000 indicates 100% germination on the first day) (three replications per treatment), according to the method of Timson (1965).

Extraction, purification, and quantification procedures. All seed tissues were lyophilized, then ground in liquid N_2 and extracted sequentially with 80% and 100%

Table 1. Germination at 20C of apple seeds stratified for 0 to 9 weeks at 5C, before and after exposure to 30C.

Stratification temp (°C)	Days at 30C	Stratification time (weeks)				
		0	3	6	9	Mean[z]
				Sum 10[y]		
20[x]	0	150 e[z]	47[w]	0[w]	0[w]	
5	0		574 b	591 b	742 a	636 r
5	3	---	308 d	373 cd	461 bc	381 s
5	6	---	38 e	88 e	78 e	68 t
Mean[z]			307 m	351 mn	427 n	
Temp treatment × stratification time			NS	NS	NS	

[z]Means followed by the same letter are not significantly different from one another within sets (a, b, c, d, e for all treatments except 3, 6, and 9 weeks at 20C; m, n for time; and r, s, t for days at 30C) by Duncan's multiple range test, $P < 0.05$.
[y]Sum 10 = cumulative percent germination for day 1 + day 2 + ... + day 10; value of 1000 indicates 100% germination on day 1.
[x]Only data for 0 weeks were analyzed statistically.
[w]Data not included in statistical analysis or means for stratification time.
[NS]Not significant at $P = 0.05$.

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Table 2. Effects of stratification temperature and subsequent exposure to 30C on ABA concentration in embryonic axes, cotyledons, and seed coats of apple seeds.

Stratification temp (°C)	Days at 30C	Dry wt (ng·g⁻¹) Stratification time (weeks) 0	3	6	9
		Embryonic axes			
20	0	106 c[z]	62 c	401 a	88 c
5	0		82 c	179 b	70 c
5	3		89 c	102 c	83 c
5	6		56 c	88 c	68 c
Temp × time			**	**	**
		Cotyledons			
20	0	26 cdef	58 b	106 a	54 b
5	0		21 def	13 f	15 ef
5	3		25 cdef	40 c	29 cde
5	6		36 cd	31 cd	26 cdef
Temp × time			**	**	**
		Seed coats			
20	0	115 cde	344 b	528 a	449 ab
5	0		188 cd	204 c	436 ab
5	3		55 e	210 c	113 cde
5	6		75 de	149 cde	160 cde
Temp × time			**	**	**

[z]Within seed tissues, means followed by the same letter are not significantly different from one another, by Duncan's multiple range test, $P < 0.05$. Means for time and stratification temperature are omitted because of interaction.
**Significant at $P = 0.05$.

Table 3. Linear correlation coefficients *(r)* of endogenous ABA concentration in apple seed tissues vs. time at various temperatures.

Treatment	Embryonic axes	Cotyledons	Seed coat
0,3,6,9 weeks at 5C	−0.093	−0.663*	0.781**
0,3,6,9 weeks at 20C	0.218	0.215	0.805**
3 weeks at 5C, then 0,3,6 days at 30C	−0.329	0.675*	−0.653
6 weeks at 5C, then 0,3,6 days at 30C	−0.755*	0.627	−0.515
9 weeks at 5C, then 0,3,6 days at 30C	−0.047	0.648	−0.709*

*,** Significant at $P = 0.05$ or 0.01, respectively (F test).

Table 4. Linear correlation coefficients *(r)* of germination capacity vs. endogenous ABA concentration in apple seed tissues.

Treatment	Embryonic axes	Cotyledons	Seed coats
0,3,6,9 weeks at 5C	−0.157	−0.619*	0.682*
0,3,6,9 weeks at 20C	−0.830**	−0.286	−0.541
3 weeks at 5C, then 0,3,6 days at 30C	0.330	−0.676*	0.650
6 weeks at 5C, then 0,3,6 days at 30C	0.733*	−0.572	0.541
9 weeks at 5C, then 0,3,6 days at 30C	0.090	−0.591	0.658

*,**Significant at $P = 0.05$ or 0.01, respectively (F test).

acetone containing 1% acetic acid and 10 mg BHT/liter (10 seed coat : 10 cotyledon and 1 embryonic axis tissue :100 solvent ratio). (±)^{3}H-ABA (≈ 5000 cpm) was added to each sample for determination of recovery. The tissues were extracted by shaking gently in darkness at 4C for 12 to 16 hr. The supernatant solution was drawn off with a Pasteur pipette and more solvent added; this was repeated twice. The extracts were dried under a stream of air and redissolved in aqueous 1% acetic acid. All samples were filtered, then purified by reverse phase HPLC on a µBondapack C_{18} (10-µm particle size), 10 × 0.8-cm cartridge column (Waters Associates, Milford, Mass.). The samples were eluted by means of a convex gradient (curve 5 on the Waters Associates Model 600 Solvent Programmer) from 0% to 50% ethanol in aqueous 1% acetic acid. The retention time of ABA was 18.5 min at a flow rate of 2 ml·min^{-1}, as determined by UV absorption of authentic (±) *cis, trans*-ABA at 254 nm. The fraction containing ABA was dried and methylated with ethereal diazomethane. Quantification of the methyl ester of ABA (Me-ABA) was performed with a Varian 3700 gas chromatography (Varian, Sunnyvale, Calif.) equipped with a ^{63}Ni-electron capture detector. Samples were dissolved in ethyl acetate containing dieldrin as an internal standard, and analyzed on a Durabond DB-1 (J&W Scientific, Folsom, Calif.) gas capillary column (30 m × 0.32 mm, film thickness 0.25 µm). Injections (1 µ1) were splitless. The injector and detector were held at a constant 250 and 290C, respectively. Initial column temperature was 60C; after injection this was increased to 165C at a rate of 48 C/rein, then increased to a final temperature of 240C at a rate of 5C/min. The column carrier gas (helium) flow rate was 1 ml·min^{-1}, and N_2 was used as the detector make-up gas with a flow rate at the detector of 30 ml·min^{-1}. Two aliquots were injected from each sample and the average value was used for statistical analysis.

Identification of ABA by gas chromatography-mass spectrometry (GC-MS). The presence of ABA in samples of each seed tissue was verified using a Hewlett-Packard 5890 Gas Chromatography (Palo Alto, Calif.) coupled to a Hewlett-Packard 5970 Mass Selective Detector (MS). Samples were purified by HPLC as described above, methylated, then dissolved in ethyl acetate and injected onto a ChromPAK (12.5 m x 0.25 mm, film thickness 0.19 µm) capillary column for GC-MS analysis. Injections (1 µ1) were on-column, with the injector port maintained at 250C, and the column carrier gas (helium) flowing at a rate of 1 ml·min^{-1}. Initial column temperature was 80C; this was immediately increased 20C/min to a final temperature of 230C. The MS was operated at an ionization potential of 70 eV with a source pressure of 6 to 7 × 10^{-5} Torr. Mass spectra were recorded between 50 and 500 mass units.

Statistical analysis. The experiment was arranged in a two-factor (three times of stratification x four temperature treatments) factorial design. Analysis of variance (ANOVA) was performed and Duncan's multiple range test was used to determine mean separation. Seeds that were neither stratified nor exposed to 30C were also compared with the factorial treatments using a separate ANOVA on data for all 13 treatments. Simple linear correlations were run to determine the relationship between endogenous ABA content and germination capacity during stratification at 5C or after exposure to 3 or 6 days at 30C.

Nonstratified seeds and those held at 20C for 3 weeks germinated very poorly; seeds held at 20C for 6 or 9 weeks did not germinate (Table 1). Stratification at 5C greatly increased germination capacity, although no difference was evident between 3 and 6 weeks (Table 1). As expected, holding chilled seeds at 30C for 3 or 6 days consistently reduced subsequent germination at 20C, the effect increasing with time held at 30C.

GC-MS of the extracts of seed coats, cotyledons, and embryonic axes confirmed the presence of ABA. Major fragments (125, 134, 162, 190 m/e) and intensities were in close agreement with those of synthetic (±) *cis, trans*-ABA (data not shown).

The concentration of-ABA in the embryonic axis was similar to that in nonstratified

seeds, regardless of treatment, with two exceptions. The concentration was considerably higher after 6 weeks at 5 or 20C (Table 2). Subsequent exposure to 30C (Table 2) decreased ABA levels in axes of seeds stratified for 6 weeks (significant linear trend, Table 3), but not in those that had been stratified for 3 or 9 weeks. This resulted in a significant temperature × time interaction.

In the cotyledons, ABA concentration was significantly higher in seeds held at 20C than in all other treatments, regardless of time of sampling (Table 2). The concentration at 5C was not significantly different from the control (nonstratified) value, although linear regression indicated a significant negative trend (Table 3). Holding seeds at 30C increased ABA content of the cotyledons (Table 2) only in seeds stratified for 6 weeks; however, linear regression was significant only in seeds stratified for 3 weeks (Table 3). Significant temperature × time interaction reflected the change in ABA content at 20C vs. lack of change in other treatments.

In seed coats, ABA concentration was again consistently higher at 20C than in nonstratified seeds or in seeds held at 5C for 3 or 6 weeks. The concentration also increased in seeds held at 5C, the increase being significant only after 9 weeks (Table 2). Linear regression indicated positive trends in ABA content at both temperatures (Table 3). Transfer of stratified seeds from 5 to 30C reduced ABA content after 3 and 9 weeks but not after 6 weeks of stratification at 5C; only seeds transferred to 30C after 9 weeks showed a significant linear decrease (Table 3). Interaction was again significant.

ABA changes were not consistently correlated with germination capacity during stratification or after exposure to 30C (Table 4), some relationships being positive, some negative.

Neither stratification at SC nor subsequent exposure to 30C greatly affected ABA content (dry-weight basis) of embryonic axes (56 to 179 ng·g^{-1}) or cotyledons (13 to 40 ng·g^{-1}). Subbaiah (1987), using GC-MS, also reported that ABA levels remained fairly constant during stratification at 5C in embryonic axes (50 to 100 ng·g^{-1}) and cotyledons (50 to 70 ng·g^{-1}) of 'Northern Spy' apple seeds. Balboa-Zavala and Dennis (1977) measured ABA levels (fresh-weight basis) during stratification at 5C in seed coats, cotyledons, and embryonic axes of 'Delicious' and 'McIntosh' apple cultivars. The dry weight of the cotyledons is • •68% and seed coats 46% of their fresh weight; therefore, levels (fresh weight) in the cotyledons (44 to 108 ng·g^{-1}) and seed coats (86 to 257 ng·g^{-1}) reported by Balboa-Zavala and Dennis were • •3-fold and 1.3- to 2-fold higher, respectively, than those observed in our study and Subbaiah's (1987). However, Balboa-Zavala and Dennis reported ABA levels for embryonic axes (1582 to 9032 ng·g^{-1} fresh weight; dry weight 56% of fresh weight) that were • •100 times greater than levels observed in our study and Subbaiah's. These very high values are probably inaccurate due to the very small samples assayed •• •37 mg fresh weight), minimal sample purification, and reduced sensitivity in quantification due to the use of a packed GC column with an extremely short retention time for ABA (1.5 rein). Artifacts could easily have affected the values obtained by Balboa-Zavala and Dennis. They extracted much larger samples of cotyledon (320 mg) and seed coat (245 mg) tissue, and the ABA values reported for these tissues are in closer agreement with those reported in our study and by Subbaiah (1987).

ABA levels were not affected by 3 or 6 weeks of stratification at SC (with one exception, axes at 6 weeks); however, a large increase occurred in seed coats from seeds stratified for 9 weeks. This result was unexpected and its significance is not known. Subbaiah (1987) found high levels of ABA in the tests and endosperm (equivalent to seed coat in our work) of non stratified 'Northern Spy' and 'Lande' apple seeds. Stratification at 5C decreased ABA levels substantially; this trend was also reported by Balboa-Zavala and Dennis (1977) in 'Delicious' and 'McIntosh' cultivars.

The same or higher levels of ABA were found in seed coats, cotyledons, and embryonic axes after 3, 6, or 9 weeks at 20C than in nonstratified seeds or seeds stratified at 5C. In contrast, Subbaiah (1987) and Balboa-Zavala and Dennis (1977) reported that ABA levels dropped significantly in seeds held at 20 or 5C. The reason for this contradiction is not known.

Levels of ABA did not change consistently in any of the seed tissues during exposure to 30C after stratification at 5C. However, because of the higher metabolic rate at 30C, a study of the turnover rates of ABA and its metabolizes would be required to fully evaluate the role of ABA in this heat-induced stress process.

In conclusion, our data do not support the hypotheses that changes in ABA content are responsible for the breaking of dormancy by chilling or for the induction of secondary dormancy by high temperature.

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