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Effects of Abscisic Acid Metabolites and Analogs on Freezing Tolerance and Gene Expression in Bromegrass (*Bromus inermis* Leyss) Cell Cultures[1]

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Optical isomers and racemic mixtures of abscisic acid (ABA) and the ABA metabolites abscisyl alcohol (ABA alc), abscisyl aldehyde (ABA ald), phaseic acid (PA), and 7'hydroxyABA (7'OHABA) were studied to determine their effects on freezing tolerance and gene expression in bromegrass (*Bromus inermis* Leyss) cell-suspension cultures. A dihydroABA analog (DHABA) series that cannot be converted to PA was also investigated. Racemic ABA, (+)-ABA, (±)-DHABA, and (+)-DHABA were the most active in inducing freezing tolerance, (−)-ABA, (±)-7'OHABA, (−)-DHABA, (±)-ABA ald, and (±)-ABA alc had a moderate effect, and PA was inactive. If the relative cellular water content decreased below 82%, dehydrin gene expression increased. Except for (−)-ABA, increased expression of dehydrin genes and increased accumulation of responsive to ABA (RAB) proteins were linked to increased levels of frost tolerance. PA had no effect on the induction of RAB proteins; however, (±)- and (+)-DHABA were both active, which suggests that PA is not involved in freezing tolerance. Both (+)-ABA and (−)-ABA induced dehydrin genes and the accumulation of RAB proteins to similar levels, but (−)-ABA was less effective than (+)-ABA at increasing freezing tolerance. The (−)-DHABA analog was inactive, implying that the ring double bond is necessary in the (−) isomers for activating an ABA response.

Exogenous application of racemic ABA [(±)-ABA] increases the freezing tolerance of axenically grown plants (Chen et al., 1983; Lang et al., 1989) and plant cell cultures (Chen and Gusta, 1983) and activates changes in protein synthesis and gene expression (Robertson et al., 1987, 1988; Lang et al., 1989; Lee et al., 1991). It is not known, however, whether (+)-ABA, (−)-ABA, or a metabolite of ABA is the primary molecule activating these processes.

ABA is catabolized to varying proportions of 7'OHABA, PA, DPA, and Glc conjugates (Loveys and Millborrow, 1984; Zeevaart and Creelman, 1988). The intermediate 8'OHABA is unstable and is not readily detected, but (+)-7'OHABA, a minor metabolite of (+)-ABA, has been isolated from bromegrass (*Bromus inermis* Leyss) cell cultures in addition to the major metabolites PA and DPA (Hampson et al., 1992). PA, the first stable product of ABA degradation, inhibits seed germination and α-amylase synthesis in barley seeds (Dashek et al., 1979; Nolan and Ho, 1988), but has no effect on cress seed germination (Gusta et al., 1992) or freezing tolerance (Reaney, 1989).

ABA analogs have proven useful for structure-activity determinations (Walton, 1983, Churchill et al., 1992) and metabolism studies (Lamb et al., 1993). A synthetic structural analog of ABA, (±)-DHABA (Oritani and Yamashita, 1982), and optically pure (+)-DHABA and its (−) enantiomer (Lamb and Abrams, 1990) were investigated for their effect on freezing tolerance and gene induction. The analog, (±)-DHABA, is not converted to PA (Lamb et al., 1993), but is active in stomatal closure (Oritani and Yamashita, 1982) and cotton cotyledon abscission (Suttle and Abrams, 1993) as well as germination inhibition of cress seeds (Gusta et al., 1992) and wheat embryos (Walker-Simmons et al., 1992). In wheat embryos, (+)-DHABA, which has the same relative stereochemistry as (+)-ABA at C1' induced expression of *dhn* (RAB), group 3 *lea*, and *Em* transcripts, but the (−) enantiomer was inactive. Metabolism studies of (±)-, (+)-, and (−)-DHABA by cultures of bromegrass have been reported (Lamb et al., 1993). The degradation product of (+)-DHABA is 8'-hydroxy-2',3'-DHABA, a stable analog of 8'-OHABA, which does not cyclize to PA as does 8'-OHABA. The racemic form of 8'-hydroxy-2',3'-DHABA was not effective at increasing freezing tolerance in bromegrass suspension cells (Lamb et al., 1993).

In the present study, we examined the effects of optically active (+)- and (−)-ABA, racemic forms of ABA, PA, 7'OHABA, and two precursors of ABA (ABA alc and ABA ald) on freezing tolerance, growth, water relations, and gene

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Abbreviations: ABA alc, abscisyl alcohol; ABA ald, abscisyl aldehyde; DEPC, diethyl pyrocarbonate; DHABA, dihydroABA; DPA, dihydrophaseic acid; LT_{50}, temperature at which there was a 50% decrease in TTC reduction compared to a nonfrozen control; OHABA, hydroxyABA; PA, phaseic acid; RAB, responsive to ABA; SSC, 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0; SSPE, 150 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA, pH 7.2; TTC, triphenyltetrazolium chloride.

expression in bromegrass suspension-culture cells. In addition, the same studies were performed using the analog (±)-DHABA and its optically pure isomers. The data indicate that whereas (+)-ABA and (−)-ABA are equally active in inducing changes in gene expression, the synthetic (−)-ABA optical isomer is not as active as the natural form of ABA in inducing freezing tolerance.

MATERIALS AND METHODS

Source of Chemicals

Racemic ABA was obtained from Calbiochem and (+)-ABA was obtained from cultures of *Cercospora rosicola* Passerini (Hampson et al., 1992). The unnatural (−)-ABA was prepared by separation of racemic methyl abscisate and subsequent hydrolysis of the (−)-ABA methyl ester (Dunstan et al., 1992). Racemic mixtures were synthesized according to published methods (Mayer et al., 1976; Abrams et al., 1990; Nelson et al., 1991). Optical isomers and the racemic mixture of DHABA were synthesized according to the procedure of Lamb and Abrams (1990). The structural formulae for all compounds are depicted in Figure 1.

Cultural Conditions and Freezing Tolerance Assessment

Experiments were conducted with a smooth bromegrass (*Bromus inermis* Leyss cv Manchar) suspension culture as previously described (Chen and Gusta, 1983). Stock cultures were grown at 25°C in the dark on a rotary shaker (80–100 rpm) in 50 mL of Erickson's medium (pH 5.8) containing 0.5 mg/L of 2,4-D and maintained by subculturing at weekly intervals (Ishikawa et al., 1990). Seven-day-old stock cultures were pooled and used as a source for these studies as described by Reaney and Gusta (1987). Two grams fresh weight of cells were transferred to 50 mL of fresh medium containing the test compounds. Freezing tolerance as determined by Ishikawa et al. (1990) was expressed as the LT_{50}. Methods for measuring growth and relative water content were as previously described (Reaney et al., 1989).

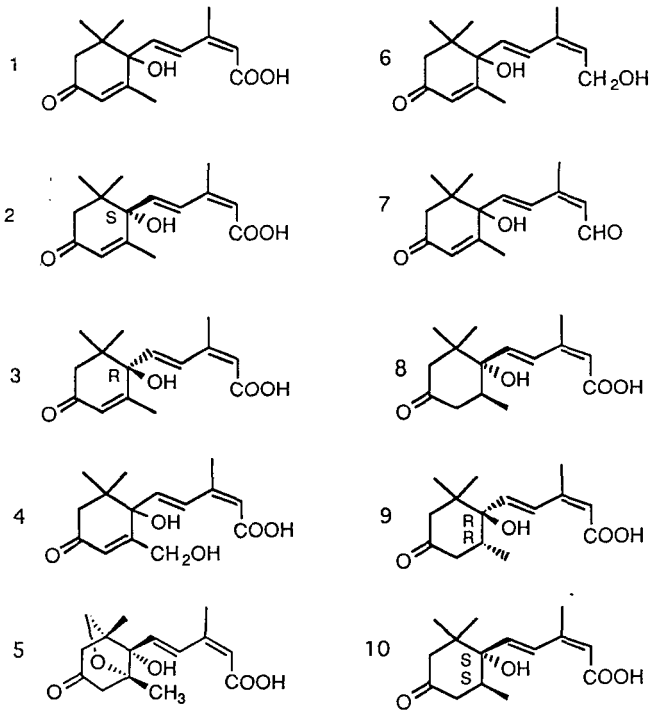

Figure 1. Structural formulae of ABA and analogs. 1, (±)-ABA; 2, (+)-ABA; 3, (−)-ABA; 4, (±)-7′OHABA; 5, (±)-PA; 6, (±)-ABA alc; 7, (±)-ABA ald; 8, (±)-2′,3′-DHABA; 9, (−)-2′,3′-DHABA; 10, (+)-2′,3′-DHABA.

HPLC Analysis of Culture Filtrates

The stability of ABA and related derivatives was examined by incubating each test compound for 7 d in Erickson's culture medium at 25°C. Media samples were stored frozen at −80°C prior to analyses. Thawed media (50 mL) were acidified with two drops of 10% HCl and extracted with ethyl acetate (20 mL, three times). Ethyl acetate extracts were pooled and re-extracted with $NaHCO_3$ solution (saturated, 20 mL, three times) and dried over anhydrous Na_2SO_4. The filtered ethyl acetate solution was dried under vacuum to form the neutral fraction. The combined bicarbonate extracts were adjusted to pH 2.5 with concentrated HCl prior to extraction with ethyl acetate (20 mL, three times). The combined organic phases were washed with NaCl solution (saturated, 20 mL, once), dried over anhydrous Na_2SO_4, and filtered, and the solvent was evaporated to yield the acid fraction.

Reversed-phase HPLC analysis of the extracted material was carried out using standard conditions (Hampson et al., 1992). The analysis of products of metabolism of ABA-related compounds in bromegrass culture filtrates was performed as described above, except that the proteins were removed by precipitation with $(NH_4)_2SO_4$ and the mixture was filtered through celite before ethyl acetate extraction.

RNA Extractions

Bromegrass cell cultures treated for 7 d with 75 μM of ABA, ABA metabolites, or ABA analogs were harvested as previously described (Ishikawa et al., 1990) and stored in liquid N_2 prior to RNA extraction. Total RNA was extracted using a modification of the procedure of Natesan et al. (1989) and quantitated by A_{260} and ethidium bromide staining. One to 3 g fresh weight of bromegrass cells were powdered in liquid N_2 prior to extraction with hot (65°C) 1 M Tris-Cl-equilibrated phenol (3 mL/g tissue). After grinding in hot phenol, an equal volume of 0.20 M sodium acetate (pH 5.2) and one-third volume of 10% SDS were added, followed by transfer to a polypropylene tube. After incubating for 5 min at 65°C, the homogenate was spun for 5 min at 5,000 rpm (Beckman J17 rotor). After transferring the aqueous phase to a clean tube, an equal volume of $CHCl_3$:IAA (24:1) was added and the tube contents were mixed and centrifuged for 5 min at 5,000 rpm. The aqueous phase was removed and transferred to a clean tube, and 2.5 volumes of 95% ethanol and LiCl (to a final concentration of 1 M) were added. The samples were then stored at −20°C for at least 2 h, and then centrifuged at 10,000 rpm for 20 min in the J17 rotor. The RNA pellet was washed once with 3 M sodium acetate and then twice with 70% ethanol, dried, and dissolved in DEPC-treated H_2O.

Northern Blotting and Hybridization

Total RNAs were separated by electrophoresis on formaldehyde gels (6% formaldehyde, 20 mM Mops, 8 mM sodium acetate, 1 mM EDTA, pH 7.2, 1.2% agarose) by running at 75 V for 3 h. The RNA was transferred to Zeta probe nylon membranes (Bio-Rad, La Jolla, CA) by capillary blotting with 20× SSC for 24 h. The RNA was fixed onto the membranes by UV irradiation for 5 min and stored in plastic bags at −20°C. The filters were prehybridized in 10 mL of hybridization solution (40% formamide, 5× SSPE, 1% SDS, 10% dextran sulfate, 5× Denhardt's solution) with 2 mg of yeast tRNA at 43°C for 6 h. Hybridization was carried out under the same conditions overnight with 2 mg of denatured yeast tRNA and 30 ng of *dhn-4* cDNA (courtesy of Dr. T. Close, University of California, Riverside, CA) labeled with [^{32}P]-dCTP using the random hexamer priming method (Feinberg and Vogelstein, 1984). The membranes were then washed twice in 2× SSPE, 2% SDS, once at room temperature, and once at 60°C. The membrane was then washed once at 60°C in 1× SSPE, 0.1% SDS.

Protein Isolation and Two-Dimensional Electrophoresis

Control and treated bromegrass cells were harvested after 7 d of culture by filtration on Miracloth and washed with distilled water (50 mL/g fresh weight). Two to 3 g fresh weight of washed cells were suspended in 6 mL/g fresh weight of 62.5 mM Tris, pH 6.8, containing 6 μL of 1.0 M PMSF, and were disrupted in a motorized ground-glass homogenizer until cell breakage approached 100%, as determined by phase-contrast microscopy. To facilitate breakage, the cells were rapidly frozen in liquid N_2 and thawed prior to homogenization. The 13,000*g* (20 min) supernatant fraction was collected for protein assay, isolation of heat-stable protein fractions, and two-dimensional electrophoresis. For isolation of heat-stable protein fractions, 1.5-mL aliquots at 1.5 to 2.5 mg/mL protein from treated and control cell supernatants were held at 90°C for 30 min. Following heat treatment, coagulated proteins were sedimented at 10,000*g* for 10 min and the heat-stable proteins remaining in the supernatant were stored in liquid N_2 for electrophoretic analyses.

Protein solvation, IEF, and two-dimensional electrophoresis were carried out as described by O'Farrell (1975) with the following modifications. Nuclease solution (0.10 mg/mL DNase I, 50 μg/mL of RNase A, 0.05 M Tris, pH 7.0, 5 mM $MgCl_2$) was added to each sample (1 μL/10 mg protein) and incubated for 10 min on ice followed by a 5-min incubation at 37°C. Nuclease-treated samples were frozen in liquid N_2, freeze dried, and dissolved in sample dilution buffer containing 9.5 M urea, 2% LKB ampholines (1.0% pH range 5–8 and 1.0% pH range 3.5–10), 5% (w/v) mercaptoethanol, and 8% (w/v) Nonidet P-40 at a final protein concentration of 5 μg/μL. Second-dimension SDS gels (10% polyacrylamide) were stained with silver (Merrill and Goldman, 1984) or with Coomassie blue R-250 (Fairbanks et al., 1971). The apparent molecular mass of unknown proteins was estimated using a Pharmacia low molecular weight calibration kit containing six standard proteins ranging from 94 to 14 kD. The pH gradients were determined as described by O'Farrell (1975). Only consistent observations of well-defined polypeptides are reported. Spots were identified as numbered polypeptides, with the number placed adjacent to the spot. Matching spots having the same isoelectric point and M_r values were assigned the same numbers.

RESULTS

Effects of (+)-ABA, ABA Metabolites, and ABA Analogs on Freezing Tolerance, Growth, and Water Content of Bromegrass Suspension Cultures

Optical isomers of ABA and racemic mixtures of ABA, ABA alc, ABA ald, and the ABA metabolites PA and 7'OHABA are illustrated in Figure 1. Optical isomers of DHABA, an ABA analog without the ring double bond, and the racemic mixture are also illustrated in Figure 1. All of the compounds were tested for their effects on the freezing tolerance, growth, and relative water content of bromegrass cells after 7 d of culture at 25°C (Table I). Racemic (±)-ABA was the most effective at increasing freezing tolerance, reducing growth, and lowering the relative cellular water content (Table I). Naturally occurring (+)-ABA was also highly effective at increasing freezing tolerance (Table I, $LT_{50} = -37.1 \pm 0.5$°C), but cells treated with (+)-ABA showed less growth inhibition and higher relative water contents than (±)-ABA-treated cells. The (−)-ABA enantiomer was less effective at increasing freezing tolerance [8°C increase compared with a 26°C increase for (±)- and (+)-ABA], but the cells treated with (−)-ABA showed a similar growth inhibition and reduction of relative water content as those treated with (+)-ABA.

The racemic form of a minor metabolite of (+)-ABA,

Table I. *Freezing tolerance, growth, and relative water content of bromegrass cells treated with ABA, ABA metabolites, and a DHABA analog*

The treatments were supplied either as optical isomers or racemic mixtures as indicated in the text. All treatments were at 75 μM for 7 d at 25°C.

Treatment	LT_{50}[a]	Growth[b]	Relative Water Content[c]
	°C	%	
Control	−11.1 ± 0.1	100 ± 7	100 ± 5
1.[d] (±)-ABA	<−40	56 ± 4	70 ± 3
2. (+)-ABA	−37.1 ± 0.5	82 ± 6	77 ± 1
3. (−)-ABA	−19.0 ± 0.2	75 ± 1	80 ± 5
4. (±)-7')OHABA	−17.0 ± 0.3	70 ± 4	ND[e]
5. (±)-PA	−9.1 ± 0.1	99 ± 7	99 ± 5
6. (±)-ABA alc	−13.8 ± 0.2	94 ± 2	88 ± 1
7. (±)-ABA ald	−19.0 ± 2.0	76 ± 6	92 ± 2
8. (±)-2',3'-DHABA	−36.5 ± 6.5	76 ± 2	82 ± 1
9. (−)-2',3'-DHABA	−14.4 ± 0.2	81 ± 3	94 ± 2
10. (+)-2',3'-DHABA	−34.5 ± 0.5	71 ± 2	74 ± 1

[a] LT_{50} = 50% killing temperature determined by TTC reduction viability assay. Values are x ± range. [b] Expressed as percent of controls, where mean net growth of controls was 5.0 g ± LSD, P = 0.05. [c] Relative water content expressed as percent of controls (5.14 g H_2O/g dry weight). Values are x ± range. [d] Numbers refer to structural formulae in Figure 1. [e] ND, Not determined.

7′OHABA, was similar to (−)-ABA in increasing freezing tolerance and reducing growth. However, racemic PA, the major metabolite of ABA, had no effect on freezing tolerance, growth, or relative water content. Two putative precursors of ABA synthesis (Zeevaart and Creelman, 1988), ABA alc and ABA ald, increased freezing tolerance by 2.7 and 8°C, respectively. ABA ald was more effective than ABA alc at increasing freezing tolerance and inhibiting growth (Table I).

The racemic mixture of DHABA increased freezing tolerance, inhibited growth, and decreased relative cellular water contents to levels similar to those of (+)-ABA-treated bromegrass cells (Table I). The (+)-DHABA isomer was as effective as the racemic mixture at increasing freezing tolerance and inhibiting growth, but was more effective at reducing cellular water. The (−)-DHABA isomer was ineffective at inducing freezing tolerance, showed less growth inhibition than the (+) form, and only slightly reduced the relative water content.

Chemical Analyses of Bromegrass Suspension Culture Medium after 7 d of Treatment with ABA-Related Compounds

The products of metabolism of the ABA-related compounds were examined by analyzing the compounds present in the medium (results not shown). As previously shown (Hampson et al., 1992), DPA accumulates in the culture medium of bromegrass cells supplied with (+)-ABA. The medium of cells fed (±)-ABA contained a mixture of predominantly DPA, with smaller amounts of PA, 7′OHABA, *trans*-ABA, and residual ABA. The filtrate of cells treated with (±)-PA contained DPA as the sole ABA metabolite. The medium of (±)-ABA alc-treated cells contained small amounts of the starting compound and its *trans* isomer. The ABA ald-treated cell filtrate contained DPA; presumably the aldehyde was converted to the acid, which was further metabolized to DPA. As previously reported (Lamb et al., 1993), cells treated with (±)-DHABA gave both enantiomers of 2′,3′-dihydro-8′-OHABA and (−)-2′,3′-DHABA-4′ alcohol, but there was no evidence of PA in the culture filtrate. The (−)-DHABA analog was metabolized to the corresponding 2′,3′-dihydro-8′-OHABA and (−)-2′,3′-DHABA-4′-diol. (+)-DHABA was converted by the bromegrass cells to the corresponding enantiomer of 2′,3′-dihydro-8′-OHABA.

The Effects of ABA, ABA Metabolites, and ABA Analogs on Expression of *dhn* (RAB) and ABA-Responsive Proteins

In bromegrass cells three different transcript sizes (approximately 0.6, 0.8, and 1.2 kb) were recognized by a barley *dhn-4* cDNA clone labeled with [^{32}P]dCTP (Fig. 2A). Bromegrass cells treated with 75 μM (±)-ABA accumulated mRNA transcripts homologous to the ABA-responsive *dhn-4* gene (Fig. 2A, lane 1). The ABA-related compounds were also investigated to determine their effects on the expression of *dhn-4* (Fig. 2A). High levels of dehydrin transcripts accumulated in response to (+)-ABA (lane 2), (−)-ABA (lane 3), (±)-DHABA (lane 8), and (+)-DHABA (lane 10). A low level of dehydrin transcripts accumulated in cells treated with (±)-7′OHABA (lane 4), racemic ABA alc (lane 6), racemic ABA ald (lane 7), and (−)-DHABA (lane 9). No dehydrin transcripts were detected in either control or PA-treated cells (Fig. 2, lanes C and 5). A photograph of the ethidium bromide-stained gel is presented in Figure 2B.

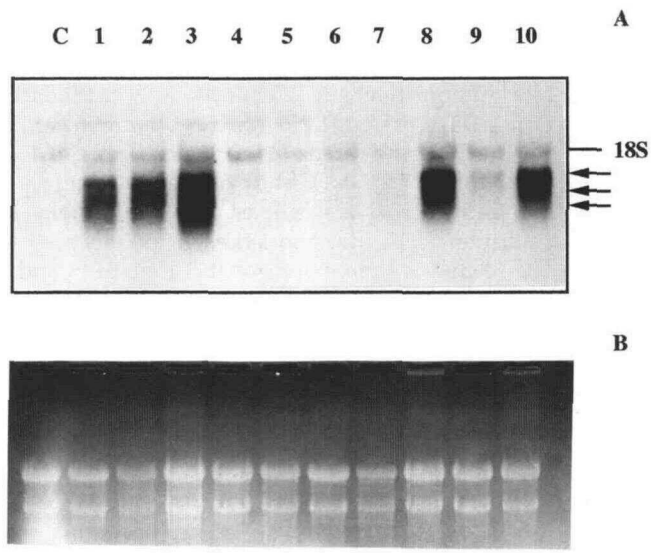

Figure 2. A, Effect of 75 μM ABA, ABA metabolites, and ABA analogs on the accumulation of dehydrin transcripts in bromegrass cells after 7 d of culture. Northern analysis of total RNA (20 μg/lane) probed with a [^{32}P]dCTP-labeled *dhn-4* cDNA. Lane designations are as follows: C, untreated control cells; 1, (±)-ABA; 2, (+)-ABA; 3, (−)ABA; 4, (±)-7′OHABA; 5, (±)-PA; 6, (±)-ABA alc; 7, (±)-ABA ald; 8, (±)-2′,3′-DHABA; 9, (−)-2′,3′-DHABA; 10, (+)-2′,3′-DHABA. Arrows depict transcripts recognized by *dhn-4* cDNA. B, Ethidium bromide-stained formaldehyde gel of RNA transferred to the membrane probed in A.

To investigate further the effects of the ABA-like compounds listed in Figure 1 on gene expression, total and heat-stable protein fractions isolated from bromegrass cells were analyzed by two-dimensional SDS-PAGE. Ninety-three silver-stained two-dimensioal gels with a minimum of three gels per treatment were analyzed, and the results are summarized in Figure 3. Racemic ABA treatment resulted in the increased accumulation of 16 major polypeptides (Fig. 3C). Thirteen polypeptides (spots 1–3, 6–10, 16, 17, 19, and 24; Fig. 3D) were heat stable, in that they resisted coagulation after heating at 90°C for 30 min. The protein patterns shown in Figure 3, C and D, for (±)-ABA were not detectably different from those for (+)-ABA, (−)-ABA, (±)-DHABA, and (+)-DHABA, except for minor variations in spot intensity. Figure 3, E and F, show the total and heat-stable protein profiles of (−)-DHABA. In silver-stained total protein profiles (Fig. 3E), only minor amounts of 9 of the 16 major ABA-responsive proteins were detected. However, electrophoresis of a heat-stable fraction isolated from (−)-DHABA-treated cells (Fig. 3F) identified the same set of heat-stable proteins responsive to (±)-ABA treatment (Fig. 3D), but their spot intensities were considerably lower. Two-dimensional patterns of protein fractions isolated from (±)-7′OHABA, (±)-ABA alc, and (±)-ABA ald were not visibly different from each other and were similar to those obtained with (−)-

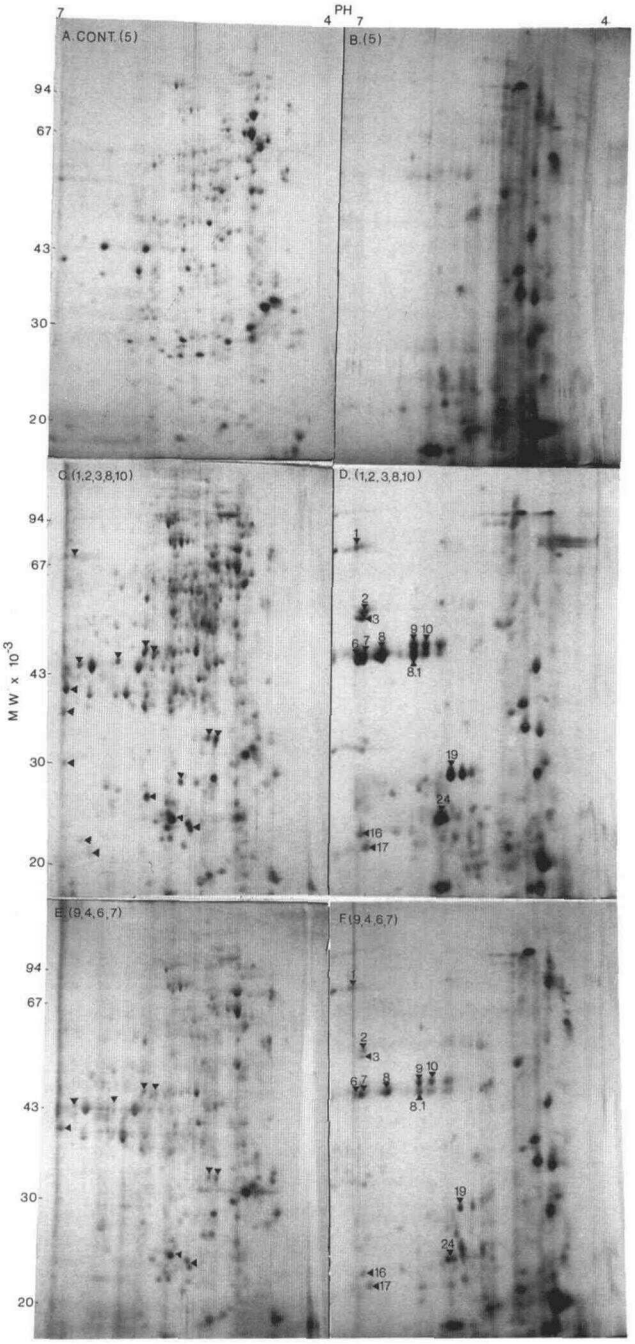

Figure 3. Comparative silver-stained two-dimensional gels of total (A, C, and E) and heat-stable (not coagulated after 30 min at 90°C) protein (B, D, F) from untreated (A, B), (±)-ABA-treated (C, D), and (−)-2′,3′-DHABA-treated (E, F) bromegrass cultures. Major polypeptide spots that were repeatedly responsive to (±)-ABA treatment are designated by solid triangles. Numbers adjacent to spots identify heat-stable (±)-ABA-responsive polypeptides not detected in untreated cells. Numbers in parentheses refer to compounds (cf. Fig. 1) used to treat bromegrass cultures that resulted in protein patterns that were not visibly different. Equal amounts (100 μg) of protein were applied to IEF gels.

DHABA (Fig. 3, E and F). The protein patterns obtained from cells treated with (±)-PA were not obviously different from those obtained from control cells (results not shown).

To confirm the results showing reduced accumulation of heat-stable ABA-responsive proteins in cells treated with (−)-DHABA, (±)-7′OHABA, (±)-ABA alc, and (±)-ABA ald, 73 two-dimensional gels stained with Coomassie blue were analyzed. For each treatment comparison, three different concentrations of protein were applied to IEF gels. These results are summarized in Figure 4. Coomassie blue R-250 staining was less effective than silver staining at detecting ABA-responsive proteins; however, Coomassie blue R-250 is considered to be more quantitative than silver staining. Similar amounts of heat-stable polypeptides were induced by (+)-ABA (Fig. 4B), (−)-ABA (Fig. 4C), and (±)-DHABA (Fig. 4E). Less RAB protein accumulated in response to (+)-DHABA than in response to the racemic form (Fig. 4F). Only trace amounts of heat-stable RAB proteins were detected in 7′OHABA-treated cells (Fig. 4D) and none were detected in (−)-DHABA-treated cells (Fig. 4G) as identified by Coomassie staining. Also, Coomassie blue staining did not detect any RAB heat-stable proteins expressed in cells treated with either ABA alc or ABA ald (data not shown).

DISCUSSION

Racemic ABA was more effective at increasing freezing tolerance, reducing growth, and decreasing relative water content than either of the individual optical ABA isomers (Table I). Although (+)-ABA was more effective at increasing freezing tolerance (LT_{50} = −37°C) than (−)-ABA (LT_{50} = −19°C), (−)-ABA was as active as (+)-ABA at decreasing growth and relative water content. The unnatural (−)-ABA has not been identified in living systems and is not active in stomatal closure (Walton, 1983); however, it inhibits growth of wheat embryos (Milborrow, 1978), barley embryos, and excised bean leaves (Walton, 1983) and induces storage protein gene expression in *Brassica napus* (Wilen et al., 1993). The degradation of (−)-ABA is much slower than that of (+)-ABA in bromegrass cell cultures (Abrams et al., 1989). In white spruce somatic embryo suspension cultures supplied with (±)-ABA, only (+)-ABA and not (−)-ABA was metabolized (Dunstan et al., 1992). The lower rate of (−)-ABA degradation, coupled with the effectiveness of (−)-ABA in inducing the dehydrin genes and RAB proteins, may explain in part why the racemic mixture shows the greatest activity in inducing freezing tolerance.

In general, there was a significant correlation between relative water contents and freezing tolerance (r^2 = 0.79, P = 0.01), decreased growth and relative water content (r^2 = 0.63, P = 0.05), and decreased growth and freezing tolerance (r^2 = 0.59, P = 0.05). There was increased expression of dehydrin genes and increased accumulation of RAB proteins in cells that showed lower relative cellular water contents. Freezing tolerance also increased with increased dehydrin gene expression and RAB protein accumulation, except in (−)-ABA-treated cells. Racemic ABA, (+)-ABA, and (−)-ABA were equally effective at increasing dehydrin gene expression (Fig. 2) and RAB protein accumulation (Fig. 3), but (−)-ABA was only partially effective at increasing the freezing tolerance of

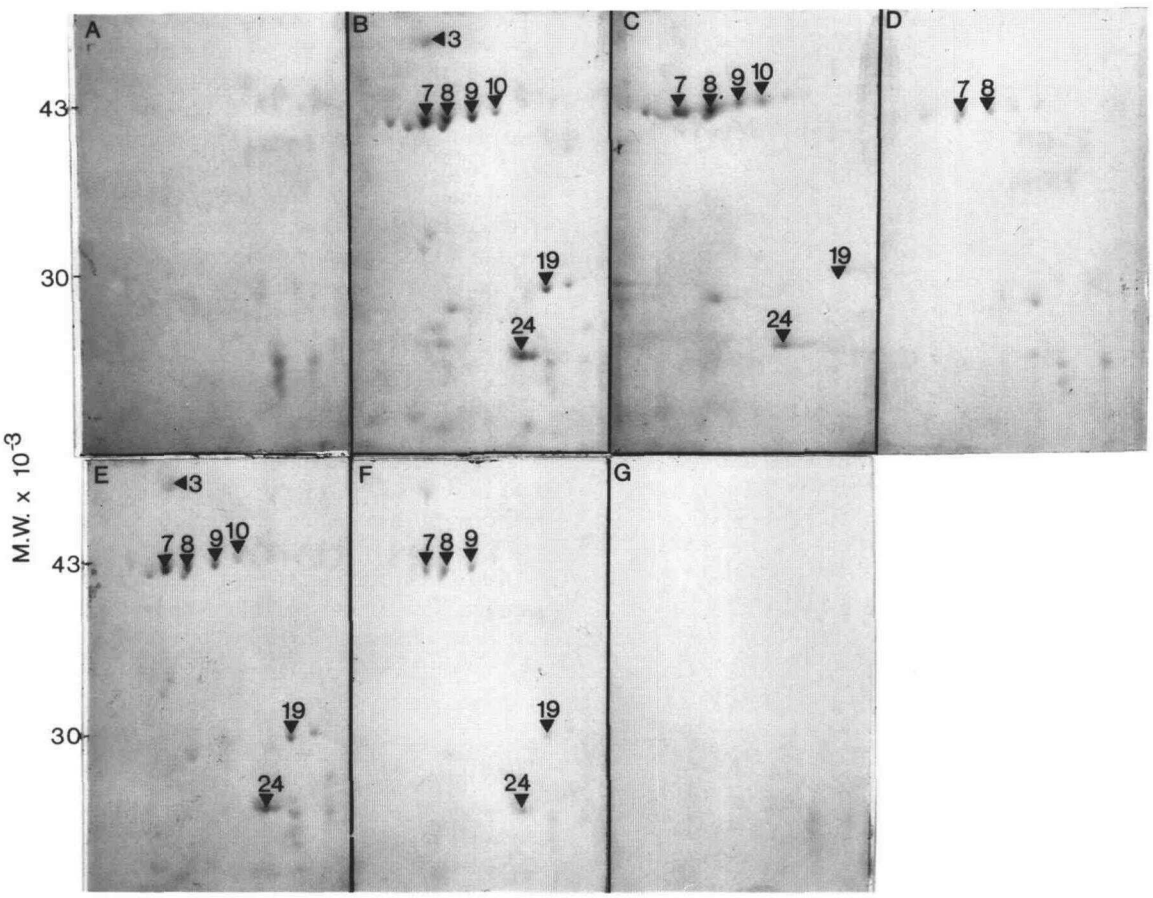

Figure 4. Coomassie blue-stained two-dimensional protein patterns of heat-fractionated (90°C) bromegrass cell proteins isolated from untreated cells (A) and cells treated with (+)-ABA (B), (−)-ABA (C), 7′OHABA (D), (±)-2′,3′-DHABA (E), (+)-2′,3′-DHABA (F), or (−)-2′,3′-DHABA (G). Only relevant sections of two-dimensional gels are shown. All IEF gels were loaded with 75 μg of protein, and the pH gradient ran from pH 7 on the left to pH 5 on the right of each panel.

the bromegrass cells. These observations suggest that (−)-ABA may be less effective than (+)-ABA in eliciting unidentified cellular responses that are required for high levels of freezing tolerance. The data also imply that increased expression of the RAB proteins induced by (+)- and (−)-ABA are correlated with and/or linked with frost tolerance but are not adequate for maximum expression of freezing tolerance. Comparison of the molecular changes occurring in these cells treated with either (+)- or (−)-ABA may reveal other important factors contributing to frost tolerance. No major differences between the protein patterns of (+)- and (−)-ABA-treated cells were detected. Also, expression of dehydrin genes in (−)-ABA-treated cells was at least equivalent to those expressed in (+)-ABA-treated cells.

In preliminary analyses of the total cellular sugar contents, control cells accumulated 4.6 times as much Glc and Fru and 30 to 50% less Suc than (±)-ABA-treated cells (results not shown). These observations are consistent with the report of Tanino et al. (1990), who demonstrated using the same cell culture that (±)-ABA treatment increased Suc uptake. Also, studies using the same culture have shown that (±)-ABA treatment results in the increased expression of genes associated with sugar metabolism (Lee and Chen, 1993). Suc also enhances the freezing tolerance of (±)-ABA-treated bromegrass suspension cultures (Reaney, 1989; Ishikawa et al., 1990). Increased Suc accumulation also occurs during cold acclimation of Puma rye (Koster and Lynch, 1992). The possible interaction between RAB proteins and solutes, such as Suc, in conferring protection against stress-induced injury of cellular proteins and membranes is currently being investigated.

ABA alc and ABA ald are biosynthetic precursors of ABA (Zeevart and Creelman, 1988). In this study, (±)-ABA alc and (±)-ABA ald showed some activity in increasing freezing tolerance, inhibiting growth, and decreasing cellular water content, as well as inducing low levels of dehydrin transcripts and RAB proteins. Analysis of the culture filtrates from these treatments at the end of the 7-d culture period revealed the presence of ABA, the metabolites DPA and C-2,C-3 *trans*-ABA alc and -ABA ald, as well as the unconsumed analogs. Previous studies have shown that most C-2,C-3 *trans*-ABA analogs are not active in inducing freezing tolerance (Churchill et al., 1992). The observed biological activity of (±)-ABA alc and (±)-ABA ald in bromegrass cell cultures may be a result of their oxidation to ABA or possibly their similarity in structure to ABA.

A minor metabolite of (+)-ABA in bromegrass cell culture filtrate, (+)-7'OHABA, was further converted to as yet undetermined substances at the end of the culture period (Hampson et al., 1992). The only acidic metabolite identified in the culture treated with (−)-ABA was (−)-7'OHABA. In this study, (±)-7'OHABA showed significant biological activity, since it increased freezing tolerance by 6°C and decreased growth of bromegrass cells by 30% (Table I). Low levels of dehydrin transcripts were expressed (Fig. 2) and RAB proteins (Figs. 3 and 4) accumulated after treatment with (±)-7'OHABA, consistent with its effect on freezing tolerance and growth.

The initial major metabolite of (+)-ABA in this bromegrass cell culture filtrate was natural (−)-PA, which was converted to DPA at the end of the culture period (Hampson et al., 1992). It is not known if (−)-ABA is converted to (+)-PA in this cell culture; however, a low rate of conversion has been demonstrated in avocado fruits (Okamoto and Nakazawa, 1993). In this study, (±)-PA had no effect on freezing tolerance, growth, or cellular water content, which is consistent with previous results (Reaney, 1989).

Although (±)-PA had no effect on gene expression in bromegrass suspension cultures, (−)-PA induced dehydrin genes in barley aleurone cells (Ariffin, 1986) and inhibited seed germination and α-amylase production in barley seeds (Dashek et al., 1979; Nolan and Ho, 1988). Both (±)-PA and (±)-7'OHABA were ineffective as germination inhibitors in cress seed (Gusta et al., 1992). These observations suggest that there may be species- and tissue-specific differences in the sensitivity to PA and 7'OHABA.

The racemic DHABA mixture and (+)-DHABA analog were as effective as (+)-ABA in increasing freezing tolerance and inducing dehydrin transcripts and proteins responsive to ABA treatment. This ABA analog can be oxidized at the 8' carbon by plant cells (Lamb et al., 1993), but because it lacks a ring double bond it cannot cyclize to PA. The activity of (±)- and (+)-DHABA confirms that PA is not necessary for ABA induction of freezing tolerance. However, (−)-DHABA was less effective than (−)-ABA in increasing freezing tolerance, reducing growth, decreasing relative water content, and inducing gene expression. The results suggest that (+)-ABA and not one of its precursors or metabolites is the major molecule in inducing freezing tolerance in this bromegrass cell culture.

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