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Response of Cultured Maize Cells to (+)-Abscisic Acid, (−)-Abscisic Acid, and Their Metabolites[1]

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The metabolism and effects of (+)-*S*- and (−)-*R*-abscisic acid (ABA) and some metabolites were studied in maize (*Zea mays* L. cv Black Mexican Sweet) suspension-cultured cells. Time-course studies of metabolite formation were performed in both cells and medium via analytical high-performance liquid chromatography. Metabolites were isolated and identified using physical and chemical methods. At 10 μM concentration and 28°C, (+)-ABA was metabolized within 24 h, yielding natural (−)-phaseic acid [(−)-PA] as the major product. The unnatural enantiomer (−)-ABA was less than 50% metabolized within 24 h and gave primarily (−)-7′-hydroxyABA [(−)-7′-HOABA], together with (+)-PA and ABA glucose ester. The distribution of metabolites in cells and medium was different, reflecting different sites of metabolism and membrane permeabilities of conjugated and nonconjugated metabolites. The results imply that (+)-ABA was oxidized to (−)-PA inside the cell, whereas (−)-ABA was converted to (−)-7′-HOABA at the cell surface. Growth of maize cells was inhibited by both (+)- and (−)-ABA, with only weak contributions from their metabolites. The concentration of (+)-ABA that caused a 50% inhibition of growth of maize cells was approximately 1 μM, whereas that for its metabolite (−)-PA was approximately 50 μM. (−)-ABA was less active than (+)-ABA, with 50% growth inhibition observed at about 10 μM. (−)-7′-HOABA was only weakly active, with 50% inhibition caused by approximately 500 μM. Time-course studies of medium pH indicated that (+)-ABA caused a transient pH increase (+0.3 units) at 6 h after addition that was not observed in controls or in samples treated with (−)-PA. The effect of (−)-ABA on medium pH was marginal. No racemization at C-1′ of (+)-ABA, (−)-ABA, or metabolites was observed during the studies.

Despite the importance of the plant growth regulator ABA to plant development and adaptation to stress, little is known about its mode of action (Hetherington and Quatrano, 1991). The role of ABA metabolism and ABA metabolites in regulating and/or mediating hormone effects is especially unclear. Much of the literature is difficult to interpret because most investigators employed racemic mixtures containing equal quantities of (+)-*S*-ABA (the natural hormone) and (−)-*R*-ABA (the unnatural enantiomer) (Fig. 1). Evidence has accumulated that these two compounds exhibit different biochemical and physiological effects and that these differences vary considerably from system to system. With respect to metabolism, the two forms of ABA are converted to different products.

In many systems, (+)-ABA is converted to (−)-PA (Fig. 1) (Sondheimer et al., 1974; Mertens et al., 1982; Vaughan and Milborrow, 1984; Zeevaart and Creelman, 1988; Dunstan et al., 1992; Hampson et al., 1992). In some cases, the PA was reduced to DPA (Fig. 1) and conjugates of the acidic compounds were formed (Sondheimer et al., 1974; Mertens et al., 1982; Vaughan and Milborrow, 1984; Hampson et al., 1992). In bromegrass cell cultures, (+)-ABA was also metabolized to a lesser extent to (+)-7′-HOABA, whereas (−)-ABA was transformed principally to (−)-7′-HOABA (Hampson et al., 1992). In tomato (+)-ABA was converted to PA and DPA-4′-β-*O*-glucoside, and (−)-ABA was converted predominantly to (−)-ABA-GE (Vaughan and Milborrow, 1984).

It has long been recognized that both (+)- and (−)-ABA are active in most physiological assays (Sondheimer et al., 1971; Walton, 1983). Both enantiomers equally inhibited barley embryo germination (Abrams et al., 1993), but the (+)-isomer was more potent than the (−)-isomer in enhancing the freezing tolerance of bromegrass cells, in inhibiting cress seed germination (Churchill et al., 1992; Gusta et al., 1992) and barley seedling growth (Sondheimer et al., 1971), and in inducing stomatal closure (Cummins and Sondheimer, 1973). Although wheat embryo germination was equally inhibited by (+)- and (−)-ABA, the enantiomers had a differential effect on gene induction. Under conditions in which the transcript coding for the Em protein was induced by (+)-ABA, (−)-ABA was much less effective in inducing the message (Walker-Simmons et al., 1992). These results suggest that the effects of ABA enantiomers should be studied separately.

Due to the scarcity of pure ABA metabolites, there have been only a few reports concerning their effects in biological assays. For example, natural PA inhibited α-amylase synthesis in GA-stimulated barley aleurone layers (Uknes and Ho, 1984). Also, racemic PA inhibited germination of cultured embryos and increased production of α-amylase inhibitor

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Abbreviations: ABA-GE, abscisic acid glucose ester; DPA, dihydrophaseic acid; EtOAc, ethyl acetate; EtOH, ethanol; HOAc, acetic acid; 7′-HOABA, 7′-hydroxyabscisic acid; *i*-PrOH, *iso*-propanol; LC/CFSIMS, liquid chromatography/continuous-flow secondary-ion mass spectrometry; MeOH, methanol; PA, phaseic acid.

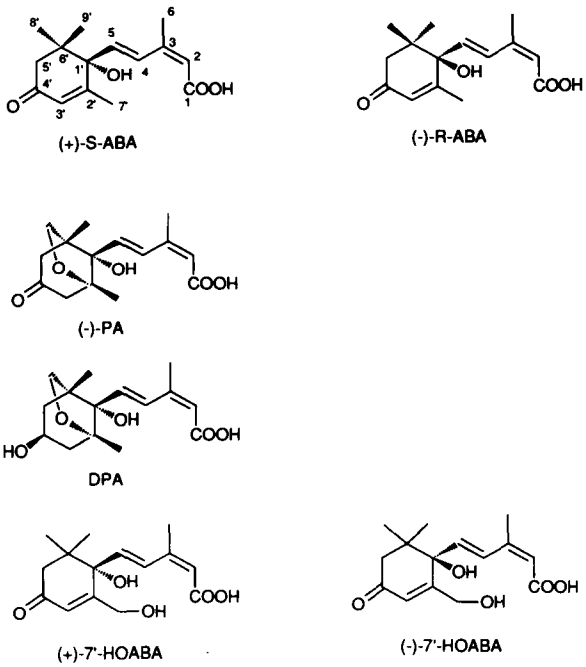

Figure 1. Molecular structures of natural ABA [(+)-*S*-ABA], unnatural ABA [(−)-*R*-ABA], and some of their known acidic metabolites.

and barley germ agglutinin (Hill et al., 1993). On the other hand, neither racemic PA nor racemic 7′-HOABA inhibited cress germination (Gusta et al., 1992), and racemic PA did not enhance freezing tolerance in bromegrass (Gusta et al., 1990). ABA metabolites were clearly active in at least some assays and therefore can potentially contribute to the in vivo effects produced by the natural hormone.

Cell-suspension cultures are convenient experimental systems for monitoring metabolism and effects of added substances. Accordingly, we screened a number of cultures for their responses to exogenous (+)-ABA. Preliminary experiments established that maize (*Zea mays* L. cv Black Mexican Sweet) suspension-cultured cells rapidly metabolized (+)-ABA to (−)-PA. In addition, the ability of these cells to respond to ABA was established by Xin and Li (1992), who showed that ABA treatment increased chilling tolerance. We describe here a detailed characterization of ABA metabolism in these cells and report the effects of ABA and metabolites on cell growth as well as on extracellular pH.

MATERIALS AND METHODS

Source of Chemicals

Racemic ABA was purchased from Sigma (approximately 99%, A-2784). (+)-ABA and (−)-ABA were obtained by preparative HPLC resolution of (±)-methyl abscisate followed by hydrolysis of the resolved esters, as previously described (Dunstan et al., 1992).

(+)- and (−)-[3′,5′,5′,7′,7′,7′]-hexatritio-ABA were prepared according to a reported procedure with some modification (Bonnafous et al., 1971): (+)- or (−)-ABA (450 mg) was added to a solution of sodium methoxide in MeOH (prepared from 0.25 g of Na and 5 mL of MeOH) followed by tritiated water (0.5 mL, 2.5 Ci). The mixture was stirred at ambient temperature for 4 d, poured into 1 M HCl (110 mL), and extracted with EtOAc (3 × 20 mL). The combined EtOAc extract was washed with water (2 × 20 mL), dried (Na_2SO_4), and concentrated in vacuo. The product was purified by preparative TLC on silica gel (toluene:EtOAc:HOAc, 25:15:2 as eluent) and crystallized from EtOAc, affording (+)-ABA with a specific activity of 1.98 GBq/mmol (54 Ci/mol) or (−)-ABA with a specific activity of 1.64 GBq/mmol (44.5 Ci/mol).

(±)- and (−)-7′-HOABA were prepared as previously reported (Nelson et al., 1991). (−)-PA was isolated from the medium of corn cell cultures by the procedure described below. ABA-GE was synthesized as previously reported (Lehmann et al., 1975; Southwick et al., 1986).

Maintenance of Cells

Suspension cultures of maize (*Zea mays* L. cv Black Mexican Sweet) were maintained as described previously (Ludwig et al., 1985). Cells were maintained at temperatures of 25° to 28°C as noted in various experiments.

Metabolism Experiments

Metabolism experiments of (+)-ABA, (−)-ABA, (−)-PA, and (−)-7′-HOABA by maize cell cultures were performed as follows. For each treatment three 25-mL flasks were inoculated with cells that were 0 to 1 d postsubculture. Typically, 0.2 to 0.25 g of cells and 10 mL of medium containing ABA or related compounds were added to each flask. ABA and other additives were introduced to the medium from a 76-mM methanolic solution. The initial concentration of ABA and related compounds in each treatment was 10 μM, with an equivalent amount of MeOH in the controls. Blanks with ABA and related compounds in cell-free media and spent media were prepared to check for compound stability. The cultures were incubated on a rotary shaker at 25°C for 4 d. The media from cultures treated with (+)-and (−)-ABA were sampled for analysis every 3 h during the first 12 h, then at 24-h intervals after the 1st d. The cultures treated with the other compounds were sampled daily.

Analysis of Culture Medium

Media from cultures that were incubated with unlabeled ABA and related compounds were analyzed by a previously reported HPLC method (Dunstan et al., 1992) with some modification. At the indicated times, three 100-μL samples were removed from each flask and transferred to 1.5-mL microcentrifuge vials. The samples were centrifuged at maximum speed (12,000 rpm, room temperature) for 3 min. The supernatant was transferred to 100-μL polystyrene microvials (Hewlett-Packard, Avondale, PA) and capped with Teflon-lined septa. The samples were briefly spun in a benchtop centrifuge to remove air bubbles and frozen at −70°C until HPLC analysis on a Supelco (Bellefonte, PA) Hisep column (15 cm × 4.6 mm i.d., 5-μm packing column preceded by a Supelco Hisep 2 cm × 4.6 mm guard). The column was

eluted at 1.50 mL min^{-1} with 1% aqueous HOAc:acetonitrile (3:1), and the eluent was monitored at 262 nm. The samples (10 μL each) were injected with an autosampler. Peaks in the chromatograms were identified by comparing their retention times with those of authentic standards. For quantitation, calibration curves were constructed using solutions containing an appropriate range of concentrations of (±)-ABA, (−)-PA, and (±)-7′-HOABA.

Metabolism Studies Using Tritiated (+)- and (−)-ABA

Tritiated (+)- and (−)-ABA were supplied to maize cell suspensions (about 60 mL) 24 h after subculture. The ABA was added as a solution in 70% EtOH (0.16 mg/20 μL) to yield an initial ABA concentration of 10 μM. The amount of cells in the suspension at time of addition was about 1.5 to 2 g fresh weight/60 mL. Experiments were performed in triplicate using a total of six flasks. The cultures were incubated at 28°C in low light at 150 rpm. Aliquots of 10 mL were collected from the various flasks at the following time intervals (after addition of radiolabeled ABA): 45 min, 3 h, 6 h, 12 h, 24 h, 30 h. Aliquots were separated into cells and medium via filtration. Cells were rinsed further with water (2 × 5 mL). The medium portions were extracted with EtOAc (3 × 10 mL). Negligible radioactivity remained in the medium after extraction. The organic extracts were backwashed with water (1 × 10 mL) and concentrated in vacuo. The residues were dissolved in EtOAc (1 mL) and analyzed by HPLC using a Hisep column and a radioactivity detector as described below. Cells were extracted with MeOH (20 mL) for 2 h at ambient temperature and removed by filtration. The cells were rinsed with MeOH (2 × 10 mL) and the MeOH extracts were concentrated in vacuo. The residues were washed with hexane (1 × 5 mL) to remove lipophilic materials and then dissolved in 0.5 mL of MeOH and analyzed via HPLC.

HPLC Analyses Using Radioactivity Detection

Using a Spectra Physics (San Jose, CA) model SP8780 autosampler, samples of appropriate size (20–100 μL) were chromatographed on a Sulpelco Hisep column (15 cm × 4.6 mm i.d., 5 mm packing). The column was eluted at 1.0 mL min^{-1} with 1% aqueous HOAc:acetonitrile (3:1). The eluate was first monitored at 262 nm and then mixed in a 1:3 ratio with Flow Scint III (Packard Instrument, Meriden, CT) prior to detection of tritium using an on-line scintillation counter (Flo-One\Beta model CR, Radiomatic Instruments and Chemicals, Inc., Tampa, FL). The amount of ABA and metabolites present in the cells or medium were determined by first calculating the percentage of radioactivity present in the cells and the medium at each time point. This fraction was then converted to nmol (by multiplying by 600 nmol, the initial amount of radiolabeled ABA added) and the amount of ABA and/or metabolite present was then determined from the appropriate radiochromatogram. The amounts obtained were the average values from three experiments.

Preparative Biotransformations of (+)-ABA by Maize Cells

A stock solution of (+)-ABA was prepared by dissolving 50 mg of (+)-ABA in 1 mL of 1 N NaOH. The required volume of stock solution was added to 50 mL of medium so that the final ABA concentration was 0, 100, 200, 300, 400, 500, 1000, or 2000 μM. The pH was adjusted to 5.8 with 0.5 M HCl. The solution was autoclaved for 25 min and 1.5 g of cells were added. Two flasks were prepared for each concentration. The cultures were incubated at room temperature on a rotary shaker at 150 rpm for 6 d. Every 24 h, two 100-μL samples were removed from the medium in each flask and frozen for HPLC analysis as described above. At the end of the culture period the cells were removed by filtration. The filtrate from flasks with the same initial concentration of ABA was combined and frozen until processed for isolation of PA and unmetabolized ABA as described below.

Identification of Metabolites

(−)-PA

(−)-PA and unmetabolized (+)-ABA were extracted from the culture filtrate by a chromatographic procedure (Gunata et al., 1985). A column of Supelco Amberlite XAD-2 resin was prepared as follows. The resin (67 g) was stirred with *i*-PrOH for several minutes and the solvent was decanted. Fresh *i*-PrOH was added and the slurry was packed into a column (3 cm diameter). The column was eluted successively with *i*-PrOH (three column heights), H_2O-*i*-PrOH (1:3, v/v) (one column height), H_2O-*i*-PrOH (1:1, v/v) (one column height), H_2O (two column heights), and 1% aqueous HOAc (three column heights). The final packed height was approximately 21 cm. The combined filtrate from the cultures incubated with 300 μM (+)-ABA was thawed and filtered through glass fiber filters (Whatman GF/B, 1.0 mm). The filtrate (approximately 100 mL) was run through the XAD column, which was then eluted with 1% aqueous HOAc (three column heights) followed by *i*-PrOH (five column heights). ABA-type compounds were present in the *i*-PrOH. The eluent was analyzed by HPLC (Hisep column) for the presence of PA. The fractions containing PA were combined and concentrated in vacuo to give a white solid residue that was purified by preparative TLC as previously described (Dunstan et al., 1992) (Silica Gel 60 GF_{254}, 20 cm × 20 cm × 1 mm, toluene:EtOAc:HOAc [25:15:2] as eluent). (−)-PA was obtained as a white solid (4.9 mg, 57% yield), $[\alpha]_D^{24} = -26.3°$ (c = 0.54, MeOH) (Kitahara et al., 1989) $[\alpha]_D^{16} = -18.0°$ (c = 0.1, MeOH). The ^1H NMR spectrum of the isolated PA (500 MHz, CD_3OD) was identical to those previously reported (Abrams et al., 1990; Dunstan et al., 1992). In a large-scale preparation of (−)-PA, a solution of 79.3 mg (+)-ABA in 1.3 mL of EtOH was added to approximately 8 to 10 g of cells in 1 L of medium. All the ABA was consumed from the medium in 3 d. A column made up of 160 g of XAD-2 resin (approximately 5 cm diameter, 16 cm height) was used for the extraction. (−)-PA (40.0 mg) was obtained in 47% yield after purification by preparative TLC.

ABA-GE

Cell extracts of incubations with nonradioactive (+)- or (−)-ABA were prepared as in metabolism experiments using tritiated ABA. The presence of ABA-GE in the extracts was

analyzed by LC/CFSIMS according to the method of Hogge et al. (1993).

(−)-7′-HOABA and (+)-PA

The identification of these metabolites was reported previously (Balsevich et al., 1994).

Growth Inhibition

Maize cell cultures were prepared as for metabolism studies. The initial concentrations of ABA and related compounds ranged from 0 to 500 μM. The control culture contained an equal volume of MeOH as the sample culture with the highest concentration of additive. The cultures were incubated on a rotary shaker at room temperature for 4 d, then the cells were separated from the medium by vacuum filtration and weighed immediately. The effect of (+)-ABA and related compounds on cell growth was determined at each concentration by calculating the percentage increase in fresh weight [(final weight × 100 ÷ initial weight) − 100]. Measurements at each concentration were performed in triplicate and the average values were normalized to a control (untreated) value of 100%.

Medium pH Measurements

Maize cell suspensions (60 mL, 1.5 g of cells) that were 24 h postsubculture were treated with (+)-ABA, (−)-ABA, and (−)-PA (0.16 mg in 20 μL of 70% EtOH). The control was treated with EtOH (20 μL). Aliquots (10 mL) were taken at 45 min, 3 h, 6 h, 12 h, 24 h, and 30 h after the additions. The pH values were measured at ambient temperature with a Radiometer pHM63 digital pH meter and the values obtained are the averages of five samplings.

RESULTS AND DISCUSSION

Metabolism of (+)-ABA

Initially, it was determined that ABA was stable in both cell-free and spent media and that ABA-related compounds were not detectable in maize cell cultures that had not been supplied with ABA. The composition of the medium of a maize cell culture incubated with 10 μM (+)-ABA over a period of 4 d is shown in Figure 2. Approximately 90% of the ABA was depleted from the medium within 24 h. Under the conditions employed, (−)-PA was the major metabolite. This was similar to the results observed on incubating (+)-ABA with somatic embryo suspension cultures of white spruce (Dunstan et al., 1992), except that the metabolism proceeded much faster with the maize cells. The total concentration of metabolites was about 8 μM after 4 d. In addition to (−)-PA, a small amount (<5%) of 8′-hydroxyABA (Milborrow, 1969) was occasionally observed (G.D. Abrams, D.L. Barton, L.R. Hogge, D.J.H. Olson, unpublished results). Details on the characterization and properties of this component will be reported elsewhere.

To study the metabolism profile in the cells as well as in the medium, time-course studies using tritiated (+)-ABA were performed. The results are summarized in Figure 3. Initially,

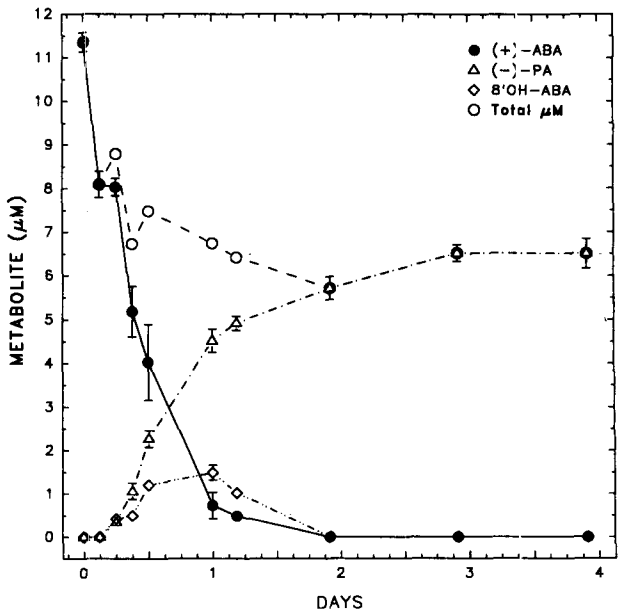

Figure 2. Time course of the metabolite profiles in the medium of a maize cell-suspension culture incubated with 10 μM (+)-ABA. Each data point represents the mean (±SD) of nine concentration determinations (three replicate cultures, concentration determination performed in triplicate for each culture).

the cells contained no radiolabeled materials. However, under the conditions employed a steady-state distribution was reached within 3 h of the addition, whereby 10 to 15% of the radioactive materials had become associated with the cells. Over the next 27 h this distribution of radioactive materials was fairly constant, even though the metabolite profile was changing.

In addition to (−)-PA, the cells accumulated conjugates that were not observed in the medium. The assignment of these materials as conjugates was based on the similarity of their HPLC characteristics with those of an authentic sample of ABA-GE. Analysis of cell extracts by LC/CFSIMS (Hogge et al., 1993) failed to confirm the presence of ABA-GE in the (+)-ABA-derived conjugate mixture but did confirm its presence in the conjugate mixture formed on feeding (−)-ABA (described below). This result was not totally surprising, since it had been reported earlier that (+)- and (−)-ABA formed different conjugates (DPA-4′-*O*-glucoside and ABA-GE, respectively) when fed to tomato shoots (Vaughn and Milborrow, 1984).

Since PA was the major metabolite in the culture medium, the maize cell culture was examined as an efficient system for the biotransformation of (+)-ABA to (−)-PA (data not shown). In a study in which the corn cell cultures were incubated with 100 to 2000 μM (+)-ABA, it was found that 500 μM was the highest concentration of (+)-ABA that could be fed to the cultures without causing cell mortality. At this initial concentration, about 8% (+)-ABA was recovered from the medium after 6 d and (−)-PA was isolated in 40% yield based on the ABA consumed. The optimum initial concentration of (+)-ABA for (−)-PA production was determined to be

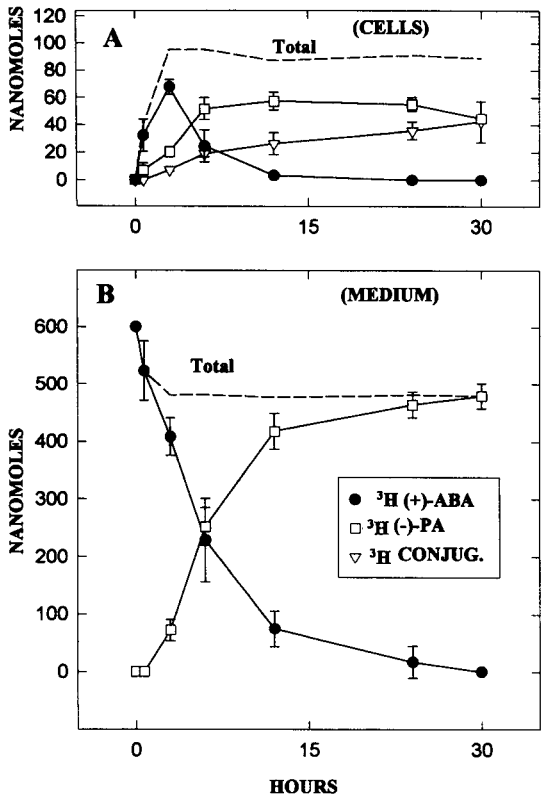

Figure 3. Time course of metabolism of tritium-labeled (+)-ABA by a maize cell-suspension culture. A, Metabolite profiles in cells; B, metabolite profiles in medium. The data at each time point are the mean (±SD) of three measurements. Experiments were conducted with about 1.5 to 2 g fresh weight cells/60 mL.

300 μM, where all the ABA was consistently consumed within 3 to 6 d and (−)-PA was isolated at 45 to 60% yield. Previous work in our facility showed that white spruce embryo suspension cultures incubated with 15 or 30 μM (+)-ABA for 8 d is a convenient preparative source of (−)-PA (Dunstan et al., 1992). The present study demonstrated that maize cell cultures are even more efficient at transforming (+)-ABA to (−)-PA. A much higher concentration of (+)-ABA was tolerated by the maize cells, a shorter incubation period was required, and a higher yield of (−)-PA was obtained.

When (−)-PA was supplied to maize cell cultures at 10 μM, its concentration in the media remained little changed over 4 d (data not shown), indicating that it was not significantly metabolized. This is consistent with the observation that the metabolite (−)-PA accumulated in the media of cultures treated with (+)-ABA.

Metabolism of (−)-ABA

(−)-ABA supplied to maize cells at 10 μM was depleted from the medium more slowly than (+)-ABA (Fig. 4). About 50% of the (−)-ABA remained unmetabolized at the end of 4 d, whereas about 90% of (+)-ABA was consumed within 24 h (Fig. 2). The major metabolite (−)-7′-HOABA was observed in the medium 1 d after addition, whereas (+)-PA was observed after 2 d (Balsevich et al., 1994).

The results with tritium-labeled (−)-ABA, summarized in Figure 5, indicated again that a steady-state distribution between cells and medium was reached within 3 h of addition, wherein about 10 to 15% of the (−)-ABA and metabolites were associated with the cells. The metabolite profiles in cells and medium, however, were quite different from those seen in the (+)-ABA treatment. The main medium metabolite was (−)-7′-HOABA, as noted above, but in the cells both (+)-PA and conjugates (mainly ABA-GE) were accumulated in larger quantities.

The biotransformation of tritiated (−)-ABA led to the production of higher amounts of (+)-PA than in the experiments with unlabeled (−)-ABA. It is not known at this time whether this result was due to an isotope effect or to differences in the experimental protocols or both. The formation of (+)-PA from (−)-ABA was noteworthy because this result has only recently been substantiated (Okamoto and Nakazawa, 1993; Balsevich et al., 1994).

The formation of conjugates associated only with the cells was again observed in the metabolism of the tritiated (−)-ABA. Analysis of the metabolite mixture by LC/CFSIMS according to Hogge et al. (1993) and comparison with an authentic sample established the presence of ABA-GE. Like (−)-PA, the metabolite (−)-7′HOABA supplied to maize cell cultures at 10 μM remained unchanged in the medium over a period of 4 d (data not shown).

Sites of Metabolism and Specificity of Enzyme(s)

The oxidation of (+)-ABA to (−)-PA took place inside the cell rather than in the medium or on the cell surface, as indicated by the observation of (−)-PA in the cells prior to

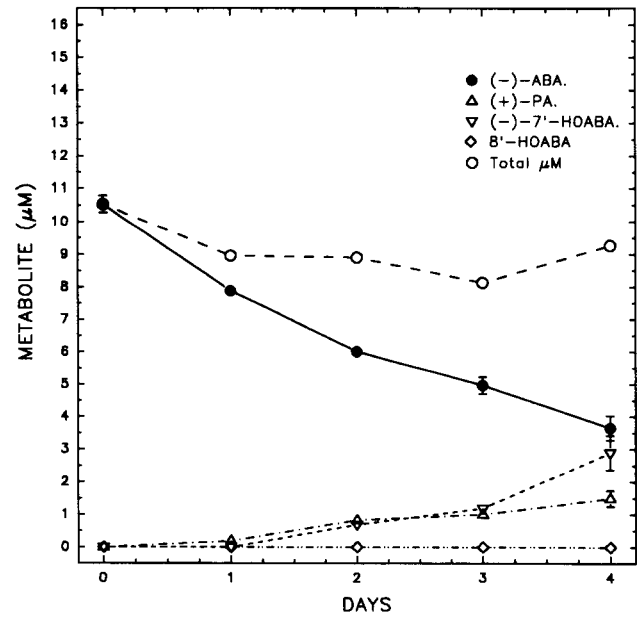

Figure 4. Time course of the metabolite profiles in the medium of a maize cell-suspension culture incubated with 10 μM (−)-ABA. Each data point represents the mean (±SD) of nine concentration determinations (three replicate cultures, concentration determination performed in triplicate for each culture).

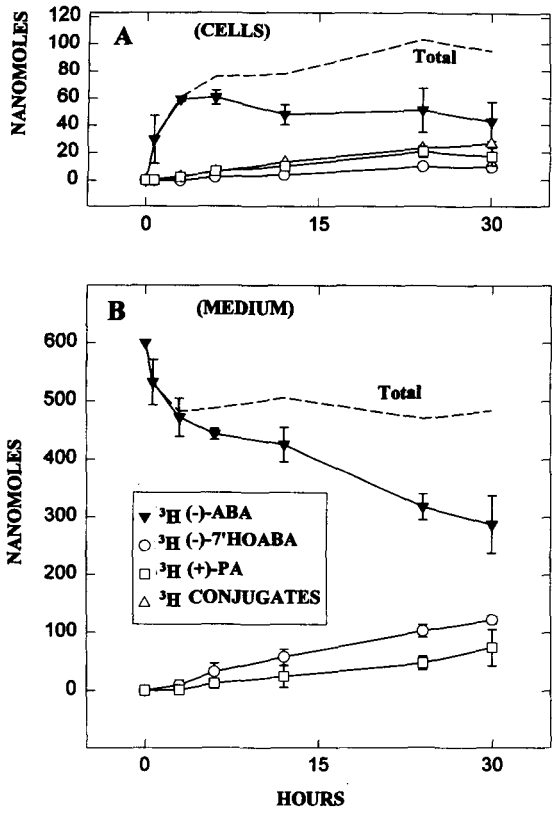

Figure 5. Time course of metabolism of tritium-labeled (−)-ABA by a maize cell-suspension culture. A, Metabolite profiles in cells; B, metabolite profiles in medium. The data at each time point are the mean (±SD) of three measurements. Experiments were conducted with about 1.5 to 2 g fresh weight cells/60 mL.

its detection in the medium and the higher proportion of PA/ABA in the cells (Fig. 3, A and B). After 45 min of incubation, (−)-PA was noted in the cells but not in the medium. After 3 h, the ratio of PA to ABA in the cells was 1:3, whereas in the medium it was 1:6; after 12 h it was 2:1 (cells) compared to 1:1 (medium). After 24 h, little ABA remained.

In contrast to the above results, in the oxidation of (−)-ABA the metabolite (−)-7′-HOABA was observed in the medium (at 3 h) before it was observed in the cells (Fig. 5, A and B). The ratio of 7′-HOABA to ABA in the medium was higher at all times than in the cells. At 6 h it was 1:14 in the medium compared to 1:26 in the cells; at 12 h it was 1:8 (medium) versus 1:12 (cells); at 24 h it was 1:3 (medium) versus 1:6 (cells); and at 30 h it was 1:2 (medium) versus 1:3 (cells). Conversion of (−)-ABA to (−)-7′-HOABA was not observed in spent medium. One explanation for these results is that the oxygenase responsible for formation of (−)-7′-HOABA is located on the cell surface (possibly cell wall bound). The 7′-HOABA being more polar would be less permeable to the plasma membrane than ABA. Therefore, its formation on the outside of the membrane would contribute to its occurrence in the medium in a higher proportion as long as the oxygenase was converting the (−)-ABA to (−)-7′-HOABA. This rationale implies that the oxygenase responsible for formation of (−)-7′-HOABA from (−)-ABA is not the same as the oxygenase responsible for formation of (−)-PA from (+)-ABA. This is consistent with the fact that enzymes normally exhibit very specific steric requirements with respect to substrates.

Previously, a "one-enzyme" hypothesis for the oxidation of (+)- and (−)-ABA to (−)-PA and (−)-7′HOABA, respectively, had been proposed (Boyer and Zeevaart, 1986; Milborrow, 1986; Loveys and Milborrow, 1991). It was suggested that (+)- and (−)-ABA could fit into an active site of the binding protein by adopting conformations that could result in the C-7′ methyl group of (−)-ABA occupying a similar space as the C-8′ methyl group of (+)-ABA. If this hypothesis were correct, (+)- and (−)-ABA would be expected to act as competitive inhibitors of each other's oxidation. In vivo competition experiments in which the rate of PA formation from (+)-ABA was monitored in the presence of an increasing concentration of (−)-ABA were performed but gave ambiguous results (data not shown). Thus, although the data from our tracer study suggested the involvement of different oxygenases in the formation of (−)-PA from (+)-ABA and (−)-7′-HOABA from (−)-ABA, conclusive proof was not obtained. Further studies, perhaps with cell-free systems, will be required to clarify this point. We are currently using the maize suspension-cultured cell system to screen for compounds that specifically inhibit the oxidation of (+)-ABA to (−)-PA, and we are investigating the characteristics of the (+)-ABA oxidizing enzyme.

The observation of conjugates of (+)- and (−)-ABA in the cells but not in the medium in this study (Figs. 3, A and B, and 5, A and B) is in agreement with earlier reports that conjugates are localized in the vacuole (Lehmann and Glund, 1986). Earlier, an enzyme preparation capable of glycosylating ABA was obtained that exhibited a pH optimum of 5.2, which is consistent with a vacuolar location (Schwarzkopf and Liebisch, 1987).

Growth Inhibition

There have been few studies that explored the relationship between ABA metabolism and the effects of ABA on cell growth and physiology. The sensitivity of this maize cell-suspension culture line to ABA has been demonstrated in previous studies in which an ABA racemic mixture induced chilling tolerance (Xin and Li, 1992). To quantify the role of ABA metabolism and metabolites, the effect of the hormone and its metabolites on cell growth was measured.

Maize cell cultures were incubated with 0 to 500 μM (+)-ABA, (−)-ABA, and their corresponding metabolites, (−)-PA and (−)-7′-HOABA. The effects of the compounds on cell growth are shown in Figure 6. (+)-ABA was the most effective inhibitor and at 1 μM it reduced cell growth to less than 50% of the control. Its metabolite (−)-PA at about 50 μM reduced cell growth to the same extent. Some inhibitory effects were observed for (−)-ABA and its metabolite (−)-7′-HOABA. Cell growth was reduced to about 50% when the cultures were treated with 10 μM (−)-ABA or with 500 μM (−)-7′-HOABA.

The growth inhibition results presented here provide another example of biological activity resulting from treatment with both optical isomers. As with some of the assays noted in the introduction, natural (+)-ABA exhibited greater po-

tency than (−)-ABA, even though the metabolism studies indicated that the (+)-isomer was present for only a short time due to its rapid oxidation. The ability of the maize cells to recognize both enantiomers [albeit with an apparently lower affinity for the (−)-isomer] is noteworthy and implies a broad substrate specificity. This is consistent with the ability of many structural analogs of ABA to produce ABA-like effects (Churchill et al., 1992; Walker-Simmons et al., 1992).

The role of ABA metabolites in mediating the effects of ABA has been unclear. In the present study, the weak effects produced by both (−)-PA and (−)-7′-HOABA eliminate both metabolic products as significant contributors to the observed inhibitions. In fact, the relative potency of (+)-ABA as a growth inhibitor versus (−)-ABA is likely to be underestimated due to its more rapid conversion to a weakly active metabolic product.

pH Effects

The results of monitoring the medium pH after addition of (+)-ABA, (−)-ABA, (−)-PA, and EtOH (control) to maize cell-suspension cultures are shown in Figure 7. The control and (−)-PA-treated samples exhibited a similar pattern in which the pH had dropped by about 0.1 pH unit after 3 to 6 h and then slowly readjusted to near the initial pH after 24 h. (+)-ABA-treated samples exhibited a dramatically different pattern in which a sharp increase of 0.3 pH units in the medium pH was observed after 6 h. The pH decreased to near the initial level over the next 24 h. (−)-ABA-treated samples exhibited a pattern intermediary to those observed for (+)-ABA-treated samples and the controls. It is also interesting to note that an increase in the total concentration of (+)-ABA and metabolites in the culture medium was observed at about the same time as the pH increase (Fig. 2).

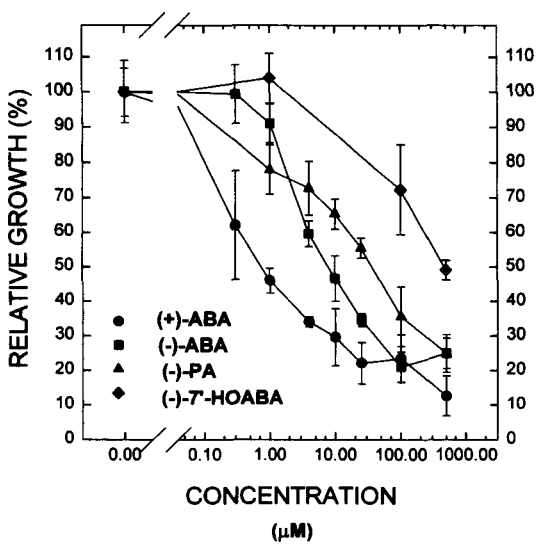

Figure 6. Effect of ABA enantiomers and metabolites on cellular growth. The effect of these compounds on cell growth was calculated at each concentration by calculating the percentage increase in fresh weight after 4 d. Measurements at each concentration were performed in triplicate and the average values (±SD) were normalized to a control (untreated) value of 100%.

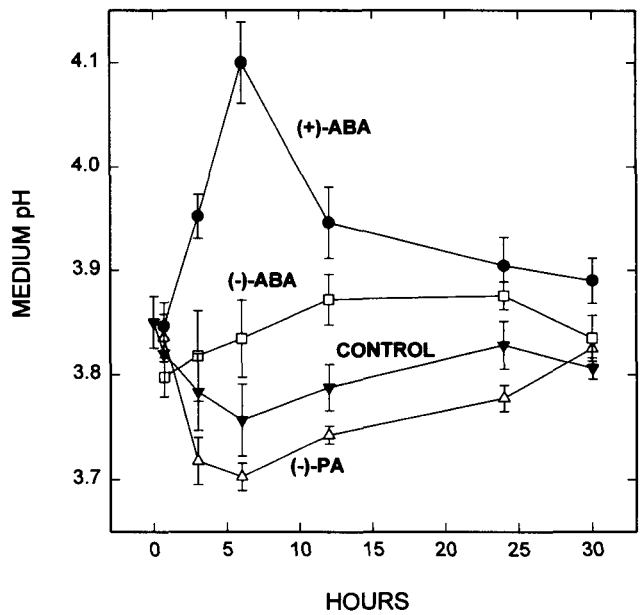

Figure 7. Effect of (+)-ABA, (−)-ABA, and (−)-PA on the medium pH of a maize suspension culture. Each data point represents the mean (±SD) of five measurements.

The lack of effect on medium pH by (−)-PA suggests the possibility that either a causal relationship between oxidative metabolism of ABA and medium pH may exist or that the pH changes are a consequence of the mode of action of ABA leading to growth inhibition. Certainly there is precedent for cytosolic pH control via metabolism (Felle, 1987), and the ability of plant growth regulators to cause transient changes in cellular pH is well documented (Gehring et al., 1990). Although there have been numerous reports on the effects of ABA on intracellular pH and Ca^{2+} fluxes, to our knowledge extracellular pH effects have not been previously documented. As well, most of the ABA-induced intracellular pH changes previously reported have been rapid events, generally occurring within 1 h, whereas the pH increase observed in this study was relatively slow. Whether the observed extracellular pH effect is general and what its physiological significance is remains to be determined.

Stereochemical Integrity

Finally, it should be noted that throughout the performance of these various studies the optical purity of the components of the metabolite mixtures were checked to see if any racemization at C-1′ had occurred. Within our detection limits, using analytical HPLC (Dunstan et al., 1992) or GC (Balsevich et al., 1994), none was observed, indicating that at least in this cell line there is no interconversion between (+)- and (−)-ABA.

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