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INDUCTION OF ABA 8′-HYDROXYLASE BY (+)-*S*-, (−)-*R*- AND 8′,8′,8′-TRIFLUORO-*S*-ABSCISIC ACID IN SUSPENSION CULTURES OF POTATO AND *ARABIDOPSIS*

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Key Word Index—*Arabidopsis thaliana*; *Solanum tuberosum*; potato; ABA 8′-hydroxylase; abscisic acid; phaseic acid.

Abstract—Suspension cultures of potato and *Arabidopsis* were incubated with 50 μM of (+)-ABA and (−)-ABA for 3 hr. These pretreatments were found to increase the rate, by two- to seven-fold, of formation of [$^2H_6$] phaseic acid (PA) from [$^2H_6$] ABA, applied in a subsequent incubation. Pretreatment with trifluoro-ABA had a higher efficacy, increasing the rate of conversion 15-fold. Suspension culture cells that had been dehydrated and then rehydrated in the presence of [$^2H_6$] ABA displayed a much lower enhancement of PA formation. We conclude that ABA induces its own oxidative catabolism in suspension cultures. ©1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The active concentration of ABA (**1**) in plant tissues is determined by the rates of its biosynthesis and catabolism. The major pathway of inactivation of ABA commences with hydroxylation (Fig. 1), yielding 8′-hydroxy-ABA (**2**) which is unstable and rearranges to phaseic acid (PA) (**3**). Cofactor requirements, $^{18}O_2$ incorporation and susceptibility to inhibitors all indicate that the ABA 8′-hydroxylase is a cytochrome P450 enzyme (P450) [1–4]. One hour after the addition of (+)-ABA to suspension cultures the rate of catabolism of absorbed substrate increases dramatically [5] and PA accumulates [6]. These data suggest that the ABA 8′-hydroxylase is activated in response to the exogenous ABA. In this study, we examined the effect of pretreatment with (+)-ABA and two ABA analogues on the formation of PA from isotopically distinct ABA supplied in a subsequent incubation. Our experimental design took into account the potentially obscuring effects of (1) different rates of absorption of substrate, and (2) different amounts of PA accumulated during the pretreatment. In previous investigations [7, 8] of this type these factors were not taken into account.

RESULTS AND DISCUSSION

In potato, both (−)-ABA and (+)-ABA stimulated PA formation seven-fold (Table 1). The ABA-treated *Arabidopsis* cultures displayed a lesser induction of activity, 2.7-fold for (+)-ABA and 2.1-fold for the unnatural enantiomer. However, the longer exposure of *Arabidopsis* to (+)-[G-$^2H_6$] ABA ([$^2H_6$] ABA) (see Experimental) may have elevated 8′-hydroxylase activity in the control. The conversion of [$^2H_6$]ABA to PA by the controls indicates that the activity is constitutively expressed. Similarly, 7.9 g of potato culture cells completely catabolized 1.2 ng of (+)-[G-^{3}H] ABA (sp. act. 0.19 μCi ng^{-1}) over 3 h (data not shown). The amounts of [$^2H_6$]PA formed were not corrected for the effects of competitive inhibition by accumulated prefed ABA. Therefore, the calculated increase is likely to be an underestimate. The magnitude of induction is similar to that reported for barley aleurone cells (two- to five-fold) [7] and germinating *Cicer* seeds (two- to four-fold) [8], both obtained with racemic ABA.

Trifluoro-ABA was more effective than (+)-ABA, increasing the rate of PA formation 15-fold (Table 1). Similarly, Todoroki *et al.* [9] found this analogue to be 30× more active than (+)-ABA in inhibiting the growth of rice seedlings, presumably because it cannot be inactivated by the 8′-hydroxylase. It is conceivable that the fluorinated compound caused accumulation of endogenous ABA by blocking catabolism, thus elevating expression of the 8′-hydroxylase. However, this would require extremely rapid turnover of the small pool of ABA in suspension cultures (ABA was not detectable in maize cell cultures [6]; less than 1 pM in potato culture medium, see below) to accumulate to

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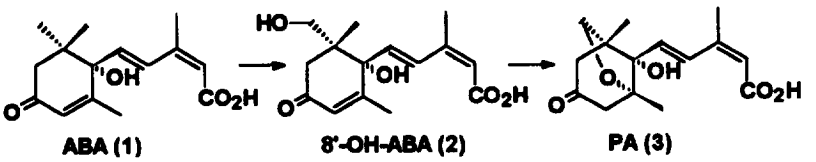

Fig. 1. Oxidative catabolism of ABA (**1**). An atom of oxygen is incorporated from molecular oxygen to give the unstable intermediate 8′-OH-ABA (**2**) which rearranges to PA (**3**).

the 1 to 10 μM required for induction of ABA 8′-hydroxylase [7].

The amount of (+)-[$^2H_6$] ABA absorbed by the cells was slightly higher in the controls (3.4 μg g^{-1} fr. wt) than in the trifluoro-ABA pretreated cells (3.1 μg g^{-1} fr. wt). This excludes the possibility that more [$^2H_6$] PA was formed by trifluoro-ABA pretreated cells due to a higher concentration of the substrate.

GC-ECD measures the entire pool of PA, including deuteriated PA depleted of label (see Experimental). Thus, the higher control value obtained by GC-ECD compared to the GC-SIM-mass spectrometry measurement is expected (Table 1). The contribution of endogenous ABA and PA (approximately 100 pg/l in the medium of potato cultures, unpublished) to the total pool was insignificant.

The rehydrated cells had a reduced viable population (51 vs 63% in the control, by vital staining) and absorbed only 31% of the substrate taken up by control cells (data not shown). Furthermore, the dehydrated cells did not regain their initial fresh weight after the 90 minute period of rehydration. Therefore, the data presented (Table 1) are expressed as ng PA formed per g viable dry wt per ng absorbed substrate. The dehydrated suspension-cultured cells displayed a slightly increased ability to produce PA from exogenous ABA. However, the effect was much less than that obtained by the ABA-pretreatments, suggesting that stress is less effective in inducing the 8′-hydroxylase activity than exposure to high concentrations of substrate (Table 1).

Table 1. Comparison of amounts of PA formed by suspension culture cells. In experiments with (+)- and (−)-ABA, PA was measured by GC-NICI-SIM-mass spectrometry, in others by GC-ECD. Data from the dehydration experiment are corrected for variations in viability and absorption of substrate. Multiple values shown are duplicates

	ng PA from [$^2H_6$] ABA g^{-1} fr. wt	
Pretreatment	*Arabidopsis*	Potato
Control (solvent)	1.3; 1.6	0.1
(−)-ABA	3.0; 3.2	0.7
(+)-ABA	4.0; 3.9	0.7
Control	2.9; 3.6	—
8′,8′,8′-(+)-Trifluoro-ABA	50.0; 50.0	—
	ng PA g^{-1} dry wt ng^{-1} absorbed [$^2H_6$] ABA	
Control	—	0.8; 0.9
Dehydrated	—	1.1; 1.1

The relevance of ABA-induction of the ABA-8′-hydroxylase to regulation of ABA concentrations in the intact plant is unknown. In detached wilted leaves the rate of formation of PA increases two-fold on rehydration of leaf blades [10, 11]. Whether this is a direct response to restoration of turgor has not been determined. If the auto-induction of the 8′-hydroxylase occurs during stress responses *in planta* then there is no need to invoke a mechanism involving the transduction of a turgor-restoration signal. Rather, the high concentrations of stress-induced ABA may stimulate an increase in expression of the enzyme, enabling rapid degradation of excess ABA as the rate of biosynthesis declines at the alleviation of stress.

The ability of exogenous and naturally-occurring compounds to induce the expression of P450s which oxidize the inducing substrate has been observed in plants and animals [12, 13]. Whitbread *et al.* [14] performed promoter analysis and found conserved elements that are required for *p*-coumaric acid inducibility of chalcone synthase in pea. The ABA response element found in many ABA-inducible genes [15] may function in an analogous manner in the ABA 8′-hydroxylase.

EXPERIMENTAL

Plant material. Suspension cultures of *Arabidopsis thaliana* Heynh. were grown in medium [16] containing 10 μM 2,4-D and maintained on an orbital shaker (150 rpm and 26°). Suspension cultures of potato (*Solanum tuberosum* L.) were prepd, with slight modifications, as described previously [17].

Chemicals. (+)- and (−)-ABA were prepd by chiral HPLC [18] of methylated (±)-ABA, followed by hydrolysis in 2 M NH_4OH at 60° for 16 hr and repurification by C_{18}HPLC (see below). [^{3}H]PA (sp. act. > 4.5 nCi pg^{-1} by GC-ECD) was obtained by feeding (±)- [^{3}H]ABA to cultures, then purifying the PA fr. (+)-[G-$^2H_6$]ABA was prepd by base-catalysed

exchange (30 min) in 0.045 M NaO^2H (99.8% atoms), then purified by C_{18}HPLC (below). ABA and analogues were redissolved in *iso*-PrOH and quantitated by UV spectrometry.

Treatment with ABA and analogues. Additives in *iso*-PrOH (0.5% of the culture vol. were supplied to 7-day-old cultures at 50 μM based on the suspension vol. Preincubations (3 hr, standard growth conditions) were terminated by filtration. Cells were washed with 5 vol. of medium, then resuspended in medium from cultures of the same age containing [$^2H_6$]ABA at 2.2 μM (*Arabidopsis*) or 9.65 μM (potato). Cultures were returned to the incubator for 20 (potato) or 60 min (*Arabidopsis*), and the second incubation terminated as above. The cell pellets were weighed and frozen with liquid N_2.

Dehydration-stress. Cells filtered from the medium were evenly spread on filter paper and exposed to air, with occasional mixing, at room temp. until 40% of the fr. wt was lost. The cells were then resuspended in medium containing 1.3 μM of [$^2H_6$]ABA and incubated for 90 min prior to harvest.

Extraction and purification of ABA and PA. A crude organic fr. was prepd by homogenizing the cells after an overnight extraction at 4° in Me_2CO–HOAc (399:1) containing 0.01% butylated hydroxytoluene (antioxidant). The slurry was filtered, then 2.27 nCi of [^{3}H] PA and 4.5 nCi of [^{3}H] ABA added to monitor recovery. This filtrate was evapd to 1–2 ml, then filtered and chromatographed on a C_{18}HPLC column (Bondclone 10, 300 mm, 3.9 mm i.d.) eluted with a linear gradient of from 99:1, H_2O–HOAc to 55:44.5:0.5 EtOH–H_2O–HOAc over 15 min (detection by UV, 260 nm). Frs corresponding to authentic ABA and PA were collected, dried, and methylated with CH_2N_2. Samples were further purified by silica HPLC (μPorasil, 300 mm, 3.9 mm i.d.) using a gradient of EtOAc in hexane (from 10 to 50% over 10 min for MeABA; 20–70% over 10 min for MePA). Frs containing MePA and MeABA were dried (N_2), then redissolved in 250 μl EtOAc containing an int. standard (MeABA for MePA measurements, endrin for MeABA). Medium was evapd to 2 ml prior to chromatography.

GC-ECD. 1 μl injections made (split ratio 10:1) onto a capillary HP-5 column. Carrier gas H_2 at 2 ml min^{-1}, operating conditions isothermal at 188° with injector temp. 260° and detector temp. 300°. Detector flushed with Ar–CH_4 (19:1, v/v) at 30 ml min^{-1}.

GC-NICI-SIM-MS. JEOL JMS-AX505H mass spectrometer used. 1μl injected onto a capillary column (DB1, 0.25 mm film thickness), carrier gas He, 1.0 ml min^{-1}. Operating conditions temp. programmed from 160 to 260° at 10° min^{-1}. Collision gases NH_4 (*Arabidopsis*) and CH_4 (potato), source pressure 2×10^{-5} torr. GC-NICI-MS (CH_4), 150 eV, *m/z* (rel. int.): 300 $[M]^-$ (100) for [$^2H_6$] MePA, 278 $[M]^-$ (98) for MeABA, GC-NICI-MS, (NH_4), 150 eV: $[M+O_2]^-$ [19] (100), 330 $[M+O_2]^-$ (100) for [$^2H_4$] MePA. The ions listed were chosen for SIM. In *Arabidopsis* experiment [$^2H_4$] PA was measured due to loss of label during storage of samples in solvent [20].

Quantitation. GC-ECD and GC-MS data were normalized to the internal standard signal, then analysed by regression against a standard curve ($r^2 = 0.995$), constructed with five different concns of the standard. Values were corrected for losses incurred during processing by measuring recovered tritium in a liquid scintillation counter. Average recovery for MePA was 58.5%. Amount of ABA absorbed was determined from the difference between the amount added and the residual ABA measured in the medium.

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REFERENCES

1. Gillard, D. F. and Walton, D. C., *Plant Physiology*, 1976, **58**, 790.
2. Zeevaart, J. A. D., Gage, D. A. and Creelman, R. A., *Plant Growth Substances* 1988, ed. R. P. Pharis and S. B. Rood. Springer, Berlin, 1990, p. 233.
3. Creelman, R. A. and Zeevaart, J. A. D., *Plant Physiology*, 1984, **75**, 166.
4. Creelman, R. A., Bell, E. and Mullet, J. E., *Plant Physiology*, 1992, **99**, 1258.
5. Windsor, M. L., Milborrow, B. V. and MacFarlane, I. J., *Plant Physiology*, 1992, **100**, 54.
6. Balsevich, J. J., Cutler, A. J., Lamb, N., Friesen, L. J., Kurz, E. U., Perras, M. R. and Abrams, S. R., *Plant Physiology*, 1994, **106**, 135.
7. Uknes, S. and Ho, T. H. D., *Plant Physiology*, 1984, **75**, 1126.
8. Babiano, M., *Journal of Plant Physiology*, 1995, **145**, 374.
9. Todoroki, Y., Hirai, N. and Koshimizu, K., *Phytochemistry*, 1995, **38**, 561.
10. Zeevaart, J. A. D., *Plant Physiology*, 1980, **66**, 672.
11. Pierce, M. and Raschke, K., *Planta*, 1981, **153**, 156.
12. Suzuki, K., Sanga, K., Chikaoka, Y. and Itagaki, E., *Biochimica et Biophysica Acta*, 1993, **1203**, 215.
13. Persans, M. W. and Schuler, M. A., *Plant Physiology*, 1995, **109**, 1483.
14. Whitbread, J. M., Thimmapuran, J. and Schuler, M. A., *Plant Physiology*, 1996, **111**, 98.
15. Ingram, J., Bartels, D., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1996, **47**, 377.

16. Murashige, T. and Skoog, F., *Physiologia Plantarum*, 1962, **15**, 473.
17. La Rosa, P. C., Hasegawa, P. M. and Bressan, R. A., *Physiologia Plantarum*, 1984, **61**, 279.
18. Railton, I. D., *Journal of Chromatography*, 1987, **402**, 371.
19. Heath, T. G., Gage, D. A., Zeevaart, J. A. D. and Watson, J. T., *Organic Mass Spectrometry*, 1990, **25**, 655.
20. Netting, A. G., Milborrow, B. V. and Vaughan, G. T., *Biomedical and Environmental Mass Spectrometry*, 1988, **15**, 375.