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The Physiological Role of Abscisic Acid in Eliciting Turion Morphogenesis[1]

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The exogenous application of hormones has led to their implication in a number of processes within the plant. However, proof of their function in vivo depends on quantitative data demonstrating that the exogenous concentration used to elicit a response leads to tissue hormone levels within the physiological range. Such proof is often lacking in many investigations. We are using abscisic acid (ABA)-induced turion formation in *Spirodela polyrrhiza* L. to investigate the mechanism by which a hormone can trigger a morphogenic switch. In this paper, we demonstrate that the exogenous concentration of ABA used to induce turions leads to tissue concentrations of ABA within the physiological range, as quantified by both enzyme-linked immunosorbent assay and high-performance liquid chromatography/gas chromatography-electron capture detection analysis. These results are consistent with ABA having a physiological role in turion formation, and they provide an estimate of the changes in endogenous ABA concentration required if environmental effectors of turion formation (e.g. nitrate deficiency, cold) act via an increased level of ABA. In addition, we show that the (+)- and (−)-enantiomers of ABA are equally effective in inducing turions. Moreover, comparison of the ABA_i levels attained after treatment with (+)-, (−)-, and (±)-ABA and their effect on turion induction and comparison of the effectiveness of ABA on turion induction under different pH regimes suggest that ABA most likely interacts with a plasmalemma-located receptor system to induce turion formation.

The plant hormone ABA has been implicated in the control of a number of events, ranging from stomatal closure to seed germination (Walton, 1980; Zeevaart and Creelman, 1988; Hetherington and Quatrano, 1991; Trewavas and Jones, 1991). We are using the duckweed *Spirodela polyrrhiza* L. as an experimental system in which ABA has been shown to induce a morphogenic response in which bud primordia are directed from their normal development (vegetative fronds) into dormant structures, termed turions (Perry and Byrne, 1969; Stewart, 1969; Smart and Trewavas, 1983a). The induction of turions by ABA has been shown to be dependent on the presence of the hormone in the culture medium during a defined period of primordium development (sensitivity window) and leads to a number of changes at the anatomical, histological, and molecular level in the responding tissue (Smart and Trewavas, 1983a, 1983b, 1984). We are using turion induction as a model system to analyze the molecular chain of events by which ABA can induce developmental switching. For example, our previous work has shown that a very early event following treatment of plants with a turion-inducing concentration of ABA is an increase in enzyme activity and transcript level of the product of a gene encoding the enzyme *myo*-inositol-3-phosphate synthase, which catalyzes the first committed step in inositol synthesis (Smart and Fleming, 1993).

However, as with any event that can be induced by the exogenous supply of a hormone, a number of questions have to be addressed before it can be accepted that the observed hormone response is physiological (Trewavas, 1991). For example, it must be shown that the exogenous concentration of hormone used to elicit the response leads to tissue levels of the hormone that are physiologically achievable by the tissue in question. This requires the quantitative analysis of internal hormone levels under both control and response-inducing conditions.

In this paper, we address the physiological nature of ABA-induced turion formation by measuring ABA levels in *S. polyrrhiza* using both ELISA and HPLC/GC-ECD analysis. These data provide us with estimates of the ABA concentration in normally growing tissue and in tissue induced to form turions by the exogenous supply of ABA. Comparison of these values allows us to judge whether the ABA level associated with turion formation is likely to be attainable by the endogenous synthesis of ABA. In addition, there has recently been much debate as to the initial site of action of ABA in eliciting cellular responses, in particular as to whether the ABA receptor mechanism is located in the cytosol (and is thus sensitive to ABA_i concentration) or faces outward into the extracellular matrix (and is thus sensitive to ABA_o concentration) (Allan and Trewavas, 1994; Assmann, 1994). We have investigated the nature of the ABA-perception mechanism for turion formation by analyzing its sensitivity to different enantiomers of

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Abbreviations: ABA_i, internal ABA; ABA_o, external ABA; ABAH, protonated ABA; $ABAH_o$, external ABAH; ECD, electron capture detection.

ABA, its sensitivity to pH, and, via a volumetric analysis based on measured values of ABA content and compartment volumes, the expected compartmental concentrations of ABA. Our data indicate that the ABA concentration required to induce turion formation is likely to be physiological, that the ABA perception system is sensitive to both (+)- and (−)-ABA, and that an external (extracellular) facing receptor seems to be the most likely site of initiation of ABA signal transduction.

MATERIALS AND METHODS

Plant Material

Spirodela polyrrhiza L. was grown aseptically on 100 mL of half-strength Hutner's medium in 250-mL Erlenmeyer flasks as described previously (Smart and Trewavas, 1983a). Each experimental flask was inoculated with two plantlets consisting of between 8 to 16 fronds. (±)-*cis-trans*-ABA and (+)-*cis-trans*-ABA were filter sterilized before addition to the medium. Cultures were harvested after 7 d and washed, and then the fresh weight of the fronds was measured and the number of vegetative fronds, turion primordia, and mature turions was recorded. The growth constants were calculated as previously described (Smart and Trewavas, 1983a). Tissue was frozen in liquid nitrogen and stored at −70°C when required for later ABA analysis.

Preparation of (+)- and (−)-Enantiomers of ABA

(±)-ABA (4 nmol plus 2.9 pmol [7.4 kBq] (±)-*cis-trans*-[^{3}H]ABA, 2.55 TBq mmol^{-1} [Amersham] to monitor the separation quantitatively) was separated into its (+)- and (−)-enantiomers by affinity chromatography using a monoclonal antibody (Mac 61) to (+)-ABA as described by Knox and Galfre (1986). The low capacity of the column required the use of repeated separations to obtain a sufficient quantity of the enantiomers for physiological studies. The (+)- and (−)-ABA-containing fractions were made pH 3.5 with HCl, partitioned three times into ether, dried under nitrogen, and purified by HPLC. Fractions containing (+)-*cis-trans*-ABA and (−)-*cis-trans*-ABA were dried under nitrogen and resuspended in methanol. The concentration of the ABA enantiomers obtained was calculated spectrophotometrically (wavelength at maximum = 252 nm; extinction coefficient = 25,200). Samples were redried under nitrogen, resuspended in medium to a concentration of 125 nM, and sterilized by filtration. One plantlet consisting of four to nine fronds was inoculated onto 4 mL of such a medium contained in a deep-sided 60-mm-diameter Petri dish. After 7 d the fronds were harvested and washed, the growth rate and turion production were monitored, and the tissue was frozen in liquid nitrogen for ABA analysis by HPLC/GC-ECD.

Extraction and Quantitation of ABA by HPLC and GC-ECD

The method used was a modification of that described by Fleming et al. (1991). Fronds (29–128 mg fresh weight, average 84 mg) were frozen in liquid nitrogen and extracted in 5 mL of methanol:water:acetic acid (80:20:0.1, v/v) containing 100 mg L^{-1} 2,6-di-*tert*-butyl-*p*-cresol with a mortar and pestle precooled to −20°C. An internal standard of 183 Bq (±)-*cis-trans*-[2-^{14}C]ABA (947 MBq mmol^{-1} [Amersham]) was added to each sample prior to homogenization. The homogenate was centrifuged at 2800*g* for 10 min and the supernatant collected. The pellet was reextracted, and the supernatants were combined. The sample was then evaporated to dryness under vacuum at 35°C and resuspended in 2 mL of dichloromethane.

The extract was loaded onto a silica Sep-Pak (Millipore) and prepurified for HPLC by the following method (adapted from Hubick and Reed, 1980). The sample was loaded onto a cartridge prewetted with dichloromethane; then after the cartridge was washed with 10 mL of acetone:dichloromethane:acetic acid (5:95:0.1, v/v), the ABA-containing fraction was eluted in 7 mL of methanol:dichloromethane:acetic acid (80:20:0.1, v/v). The eluate was evaporated to dryness under vacuum at 35°C and then resuspended in 1 mL of methanol:water:acetic acid (10:90:0.1, v/v).

HPLC was based on the method of Durley et al. (1978). An Altex Ultrasphere ODS column (Anachem, Luton, UK) was used with an SP8700 solvent delivery system, output was monitored with an SP8300 UV detector (254 nm), and traces were recorded and analyzed by an SP4100 computing integrator (Spectra Physics, St. Albans, UK). All solvents were HPLC grade. Samples (1 mL) were loaded and an isocratic gradient of methanol:water:acetic acid (10:90:0.1, v/v) was run for 10 min, followed by a gradient from 40:60:0.1 (v/v) to 70:30:0.1 (v/v) methanol:water:acetic acid over 30 min. (±)-*cis-trans*-ABA eluted as a single peak at a retention time of 24.5 min.

The ABA-containing fractions were dried under nitrogen at 55°C, resuspended in methanol, and then methylated with an excess of ethereal diazomethane (de Boer and Backer, 1963). Methylated fractions were redried under nitrogen and resuspended in 500 μL of methanol.

The ABA content of 1-μL aliquots of sample was measured using a gas chromatograph with a ^{63}Ni electron capture detector by a method based on that of Lachno et al. (1982). A 0.9-m column of 1% (w/v) Carbowax 20M on Gas Chrom Q (Phase Separations, Clwyd, UK) was used with a detector temperature of 250°C and column and injector temperature of 185°C. Oxygen-free nitrogen at a flow rate of 40 mL min^{-1} was used as the carrier gas. A standard of (±)-*cis-trans*-methyl-ABA ran as a single peak with a retention time of 15.9 min. A standard curve was produced over the range 0.01 to 50 pmol μL^{-1} methyl-ABA. After GC-ECD, 250 μL of each sample were counted for ^{14}C. Approximately 57 Bq were counted per sample against a background of about 0.3 Bq. The mean recovery was 53.0 ± 2.5% (n = 30) and there was no effect of ABA_o concentration on recovery.

Extraction and Quantitation of ABA by ELISA

The method used was a modification of that described by Walker-Simmons (1987). Fronds (33–171 mg fresh weight) were extracted in 4 mL of methanol:water:acetic acid (80:20:0.1, v/v) containing 100 mg L^{-1} 2,6-di-*tert*-butyl-*p*-cresol

as described above, except that the extract was mixed on an overhead shaker for 24 h at 4°C before centrifugation at 10,000*g* for 15 min at 4°C. An internal standard of 92 Bq (±)-*cis-trans*-[^{3}H]ABA (2.9 TBq mmol^{-1}; Amersham) was added to each sample prior to homogenization. The supernatant was dried under nitrogen at 55°C, resuspended in 2 mL of buffer (50 mM Tris-HCl, 150 mM NaCl, 2.1 mM $MgCl_2$, pH 7.8), and filtered through nylon as described by Dunlap and Guinn (1989). Recovery was calculated by counting 250 μL of each sample for ^{3}H and was found to be 77.3 ± 0.8% (n = 36). Aliquots (20–30 μL) of the samples were assayed by an indirect ELISA according to the method of Walker-Simmons (1987) using an ABA-4′-BSA conjugate prepared according to the method of Weiler (1980) and a monoclonal antibody purchased from Idetek (Copenhagen, Denmark). A standard curve was produced over the range 0.01 to 2.5 pmol (+)-ABA and several dilutions of the sample extracts were checked for possible interference using the method of internal standardization as described by Pengelly (1986) and Wang et al. (1986).

Statistical Analysis

The significance of the differences between sample means was calculated using the t test or paired t test as described by Bishop (1981). Means are expressed ± SD.

RESULTS

ABA Levels in *S. polyrrhiza* during ABA-Induced Turion Formation

To analyze the levels of ABA in *S. polyrrhiza*, we developed an ELISA for the detection of (+)-ABA in crude extracts, based on the method of Walker-Simmons (1987). The results of this analysis are shown in Figure 1A.

Control fronds, with no exogenous ABA in the medium, had a mean ABA_i level of 75 pmol g^{-1} fresh weight (±13, n = 3). Fronds incubated for 7 d in medium containing (+)-ABA (ranging from 1–5000 nM) showed an ABA_i that generally increased with increasing concentration of ABA_o. This increase of ABA_i with ABA_o was first detected at an ABA_o of about 75 nM and became statistically significantly different from control values of ABA_i at an ABA_o value of 250 nM ($P < 0.01$). ABA_i reached values of more than 7246 pmol g^{-1} at ABA_o levels of 2500 nM or higher.

The boxed area in Figure 1A indicates those values of ABA_o at which turions were induced (i.e. 250-1000 nM). There is a significant difference between ABA_i values at ABA_o = 100 nM (no turions formed) and ABA_o = 250 nM (turions produced) ($P < 0.02$). Turion-inducing ABA_o values led to ABA_i mean values ranging from 1010 ± 161 to 3203 ± 404 pmol g^{-1}. These induced ABA_i levels compare to a resting, control mean level of 75 pmol g^{-1}.

The data from Figure 1A have been plotted in Figure 1B to reveal the number of turions formed at different measured values of ABA_i. These data show that there is a window of ABA_i level within which turion formation occurs (798–3927 pmol g^{-1}) but also that in two samples (of 13) ABA_i concentrations were attained within this window but no turion formation was observed. These two samples,

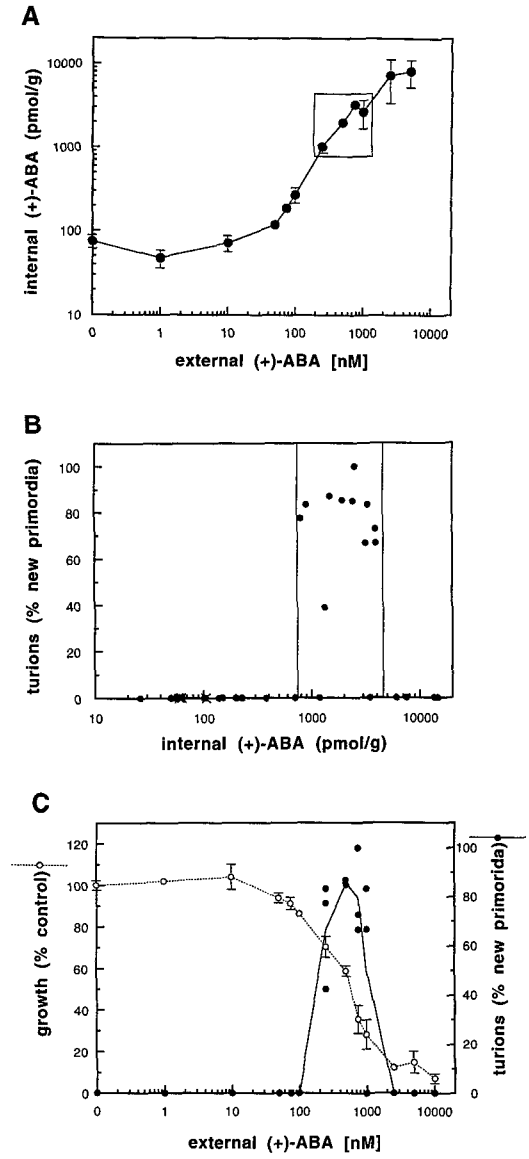

Figure 1. Correlation of internal and external (+)-ABA levels with growth and turion formation in *S. polyrrhiza*. Triplicate cultures of fronds were incubated for 7 d in concentrations of (+)-ABA ranging from 1 to 5000 nM. The number of turions and turion primordia was recorded and the (+)-ABA content was measured by ELISA. A, Internal (+)-ABA concentration (expressed as pmol g^{-1} fresh weight) achieved in the fronds after growth in external (+)-ABA. The boxed area encloses the samples that produced turions. Each point represents the mean (±SD) of three samples. Where not shown the error bars are smaller than the symbols used. B, Correlation between the extent of turion formation and the internal (+)-ABA concentration. Turion formation is expressed as the number of turions and turion primordia as a percentage of all new primordia formed during development. ●, ABA-treated samples; ×, control untreated samples. C, Correlation between turion formation and growth and the external (+)-ABA concentration. Growth (○) is expressed as the frond number growth constant k expressed as a percentage of the control, and each data point is the mean (±SD) of three samples. Where not shown, the error bars are smaller than the symbols used. Turion formation (●) is expressed as the number of turions and turion primordia as a percentage of all new primordia formed during development. The individual data points are shown.

in which turion-associated levels of ABA_i were attained without accompanying turion formation, represent tissue treated with high (supraoptimal) concentrations of ABA with respect to turion formation.

Finally, in Figure 1C the number of turions formed at various values of ABA_o has been plotted, along with the frond number growth constant under these conditions. These results show that turions were induced at ABA_o values of between 250 and 1000 nM (equivalent to the boxed region in Fig. 1A) and that in all samples incubated at an ABA_o within this window turions were formed (with the exception of one of the three samples incubated at the highest turion-inducing ABA concentration). In addition, at these turion-inducing concentrations of ABA, growth of the fronds was impaired, corroborating previous data (Smart and Trewavas, 1983a).

The specificity and accuracy of the ELISA for ABA measurements in extracts of *S. polyrrhiza* were checked both by standard internal controls (recoveries of added labeled ABA, parallelism of standards in diluted extracts [Pengelly, 1986; Wang et al., 1986; data shown in Chaloupková, 1995]) and by comparison of the values calculated by ELISA with those obtained using an independent HPLC-linked GC-ECD analysis of tissue extracts, as described in the next section.

Enantiomers of ABA Undergo Different Rates of Accumulation but Are Equally Effective in Turion Induction

To substantiate the accuracy of the estimates of ABA_i obtained via ELISA, we performed a number of measurements of ABA_i in extracts of *S. polyrrhiza* using an HPLC-linked GC-ECD method (Fleming et al., 1991). In addition, in contrast to the ELISA, the HPLC/GC-ECD method allowed us to assay for both (+)- and (−)-ABA and thus to investigate whether there was a difference in the accumulation of ABA after feeding tissue with either the mixed enantiomers or the purified (+) form. An example of such an analysis is shown in Figure 2.

These data indicate that when (+)-ABA was fed at turion-inducing concentrations (in this experiment ABA_o = 100–750 nM; 1,000 nM was not tested) the values of ABA_i measured were in the range 717 to 1,700 pmol g^{-1}. These can be compared with the data obtained from the ELISA analysis (Fig. 1A), in which ABA_i values of 798 to 3,923 pmol g^{-1} were obtained at similar turion-inducing values of ABA_o. When mixed enantiomers of ABA were supplied in the medium (ABA_o = 100–1,000 nM), ABA_i values in the range 1,702 to 15,521 pmol g^{-1} were obtained from cultures producing turions (boxed area). At lower values of ABA_o, e.g. 0 to 10 nM, concentrations of ABA within the tissue of 578 to 737 pmol g^{-1} were calculated.

The data shown in Figure 2 suggested that the different enantiomers of ABA were not accumulated at the same rate. To examine this hypothesis, we purified (+)- and (−)-ABA from the commercially available mixed enantiomers using an immunoaffinity column (Knox and Galfre, 1986) and then tested these enantiomers both for their accumulation in tissue after being fed to fronds via the

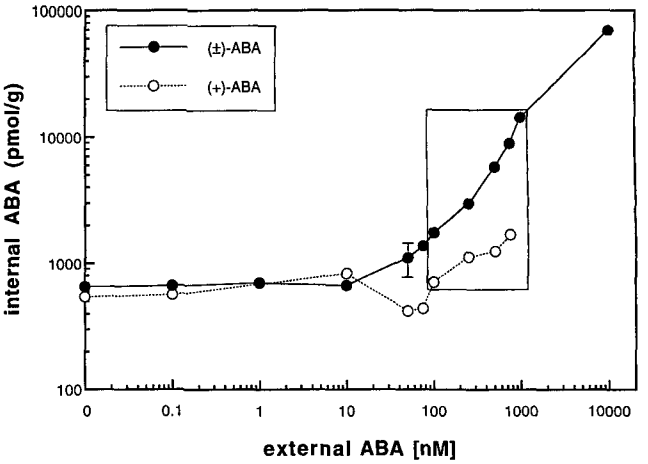

Figure 2. ABA_i concentration achieved in the fronds after growth in external (±)- and (+)-ABA. Cultures of fronds were incubated for 7 d in concentrations of (±)-ABA ranging from 0.1 to 10,000 nM. The number of turions and turion primordia was recorded, and the ABA content was measured by HPLC/GC-ECD and expressed as pmol g^{-1} fresh weight. The boxed area encloses the samples that produced turions. Each data point for (±)-ABA (●) represents the mean, and the error bars represent the range of values from two separate experiments. Where not shown, the range is smaller than the symbols used. Only one experiment was performed for (+)-ABA, using eight different concentrations between 0.1 and 750 nM (○).

medium and for their effectiveness in inducing turion formation and inhibiting growth. Because of the low capacity of the immunoaffinity column, we could only prepare small quantities of the (−)-ABA enantiomer, but the results from the single experiment that was possible are clear. Figure 3A shows the levels of ABA_i accumulated in tissue treated with a 125 nM concentration of (±)-ABA, (+)-ABA, or (−)-ABA. The ABA_i attained when (−)-ABA was fed (3838 pmol g^{-1}) was almost 4 times the value obtained with (+)-ABA alone (781 pmol g^{-1}), whereas the (±) mixture of enantiomers led to an intermediate level of ABA_i (1540 pmol g^{-1}).

The concentration of ABA_o used in this experiment (125 nM) was sufficient in all cases to induce turion formation, and the number of turions formed was similar irrespective of whether the individual or mixed enantiomers were fed (Fig. 3B). In addition, the inhibition of growth brought about by the different enantiomers at the same concentration was comparable, as shown in Figure 3B.

The effectiveness of (−)-ABA in inducing turion formation and growth inhibition was also indicated by a series of experiments in which (+)- and (±)-ABA were compared for their ability to affect these two parameters. As shown in Figure 4A, ABA at a concentration of 100 nM to 1000 nM induced turions regardless of whether the (+)- or (±)-enantiomers were used, and a statistical analysis of turion induction revealed no significant difference between the efficacy of the two treatments (mean difference 5.70 ± 10.35%, n = 26, paired t test). Moreover, 100 nM (±)-ABA [which should contain 50 nM (+)-ABA] always induced turion formation in this series of experiments, whereas 50 nM (+)-ABA alone never led to turions.

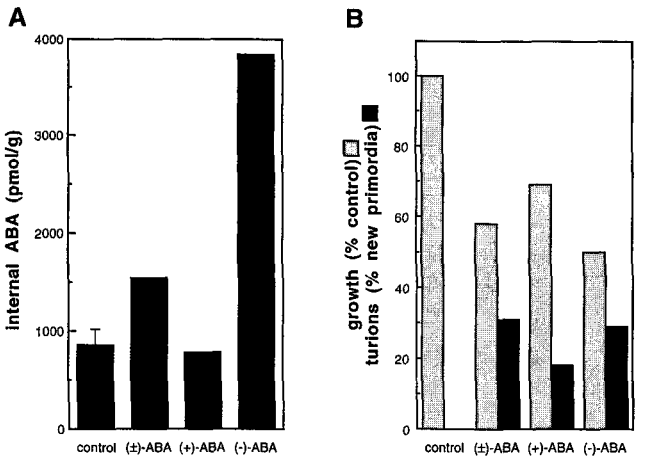

Figure 3. The effect of 125 nM (±)-, (+)-, and (−)-ABA on the ABA_i level and the extent of turion formation and growth inhibition achieved after 7 d. Commercial (±)-ABA was separated into the (+)- and (−)-enantiomers by immunoaffinity chromatography and compared for effectiveness in inducing turion formation and inhibiting growth. ABA_i levels were measured by HPLC/GC-ECD. A, ABA_i levels achieved in the fronds expressed as pmol g^{-1} fresh weight. The error bar represents the range of two samples for the control mean. B, Turion formation (■; expressed as the number of abscised turions as a percentage of all new primordia formed during development) and growth (gray column; expressed as the frond number growth constant *k* as a percentage of the control).

Similarly, an analysis of growth inhibition by (+)- and (±)-ABA, shown in Figure 4B, indicated that both treatments were equally effective in decreasing frond growth. A statistical comparison of the two treatments over the linear range of growth inhibition response from 50 to 1000 nM (mean difference −2.56 ± 3.27%; n = 32, paired t test) indicated no significant difference in the efficacy of (+)- or (±)-ABA in the inhibition of frond growth. When we performed a similar statistical analysis based on the assumption that (−)-ABA is inactive and that a defined (±)-ABA concentration contains (+)-ABA at half that concentration, we found that the mean difference in growth between (+)-ABA alone and (+)-ABA in the presence of (−)-ABA was significant at −14.87 ± 6.60% (P = 0.05, n = 15), showing that the assumption was incorrect and that (−)-ABA is active.

ABA-Induced Turion Formation Is pH Dependent

ABA is an amphipathic molecule with a pK_a of approximately 4.8. At low pH the molecule exists mainly as ABAH, which is relatively membrane permeable, whereas the ionized form (ABA^-), which is relatively membrane impermeant, predominates under alkaline conditions (Kaiser and Hartung, 1981). This difference in membrane permeability at different pHs has been used as a diagnostic tool to infer the site of action of ABA in the stomata-closure response, ABA activity when fed to cells at high pH being indicative of an outward (extracellular) facing site of ABA perception (Hartung, 1983). Therefore, we analyzed the effect of ABA_o on turion formation at various values of medium pH. The results are presented in Figure 5A.

These data show that, as the pH of the medium was increased from 4 to 7, there was a gradual increase in the value and width of the optimum ABA_o required to elicit turion formation. Concomitantly, there was an increase in the threshold value of ABA_o required to induce turion formation at all. Thus, at a pH of 4, there was maximal turion formation at ABA_o = 75 nM with a threshold of 25 nM, whereas at pH 7 the threshold was increased to 500 nM with an optimum turion induction at 1000 nM ABA. Growth inhibition by ABA showed a similar pH dependence, a greater inhibition occurring at a lower pH for a

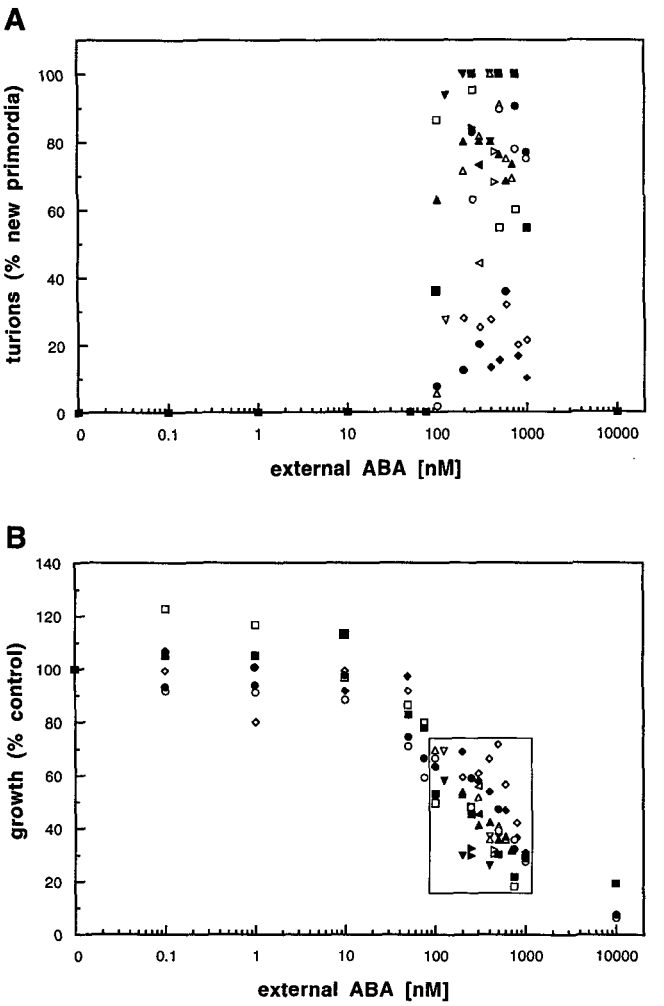

Figure 4. The effect of external (±)- and (+)-ABA concentration on turion formation and growth after 7 d. Data are from seven separate experiments. A different symbol is used for each experiment. Within each experiment, a set of cultures was treated with either (±)-ABA (filled symbols) or (+)-ABA (open symbols). A, Turion formation expressed as the number of turions and turion primordia formed as a percentage of all new primordia formed during development. B, Growth expressed as the frond number growth constant *k* as a percentage of the control. The boxed area encloses the samples that produced turions.

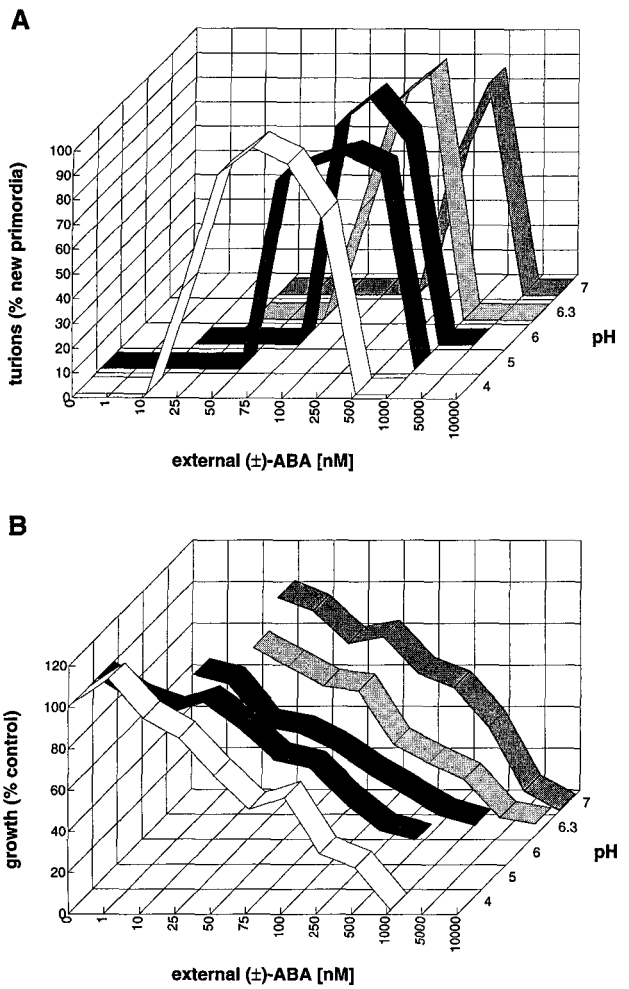

Figure 5. The effect of medium pH on the efficacy of external (±)-ABA on the induction of turion formation and inhibition of growth after 7 d. A, Turion formation expressed as the number of turions and turion primordia as a percentage of all new primordia formed during development. B, Growth expressed as the frond number growth constant k as a percentage of the control.

given value of ABA_o (Fig. 5B). Incubation of fronds at different pH values in the absence of exogenous ABA did not lead to turion formation.

As pH is increased, a decrease in the external ABAH concentration is expected, leading to a reduced flux of ABA across the plasmalemma (Rubery and Astle, 1982). If response to ABA depended only on uptake (and therefore the level of $ABAH_o$), then all points of a plot between response and $ABAH_o$ should lie on a common line, independent of the actual pH value. If the amounts of ABAH present at the various values of ABA_o and pH used in the experiments presented in Figure 5A are calculated and plotted against the number of turions formed, then the relationship between $ABAH_o$ concentration and turion formation is revealed, as shown in Figure 6A. These data indicate that, although there is a window of $ABAH_o$ values within which turion formation is observed, this window is very wide (3–300 nM) and, moreover, there are many instances in which an $ABAH_o$ concentration associated with turion formation at one pH value is not associated with turion formation at another pH value. For example, at pH 5 or lower, 50 nM $ABAH_o$ is always associated with turion formation; yet at pH 6 or higher, this same value of $ABAH_o$ never leads to the production of turions. A lack of correlation between $ABAH_o$ level and frond growth inhibition is also observed, with the response depending not only on the $ABAH_o$ concentration but also on the actual pH value (Fig. 6B).

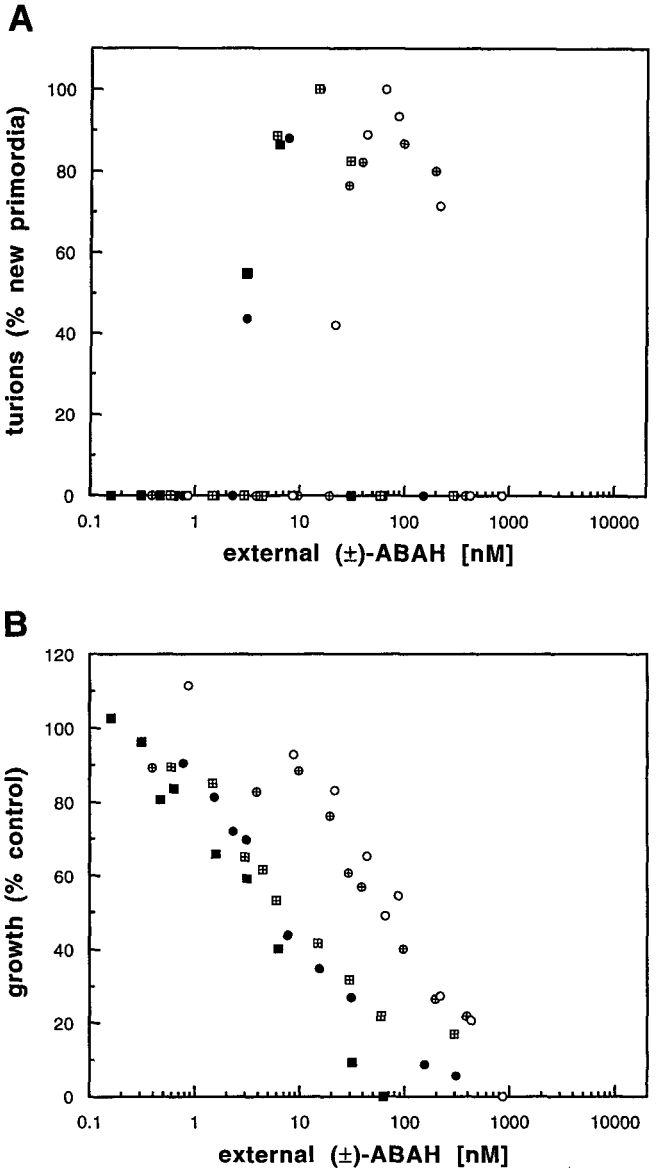

Figure 6. The relationship between external (±)-ABAH concentration and the extent of turion formation and growth after 7 d. The concentration of ABAH present at each combination of ABA concentration and pH was calculated from the data of Figure 5. ○, pH 4; ⊗, pH 5; ⊠, pH 6; ●, pH 6.3; ■, pH 7. A, Turion formation expressed as the number of turions and turion primordia as a percentage of all new primordia formed during development. B, Growth expressed as the frond number growth constant k as a percentage of the control.

DISCUSSION

ABA Levels Used to Induce Turion Formation Are Physiological

The induction of turions by ABA in *S. polyrrhiza* presents a unique system for the analysis of a developmental switch in a plant effected by a hormone. The alternate phenotypes (vegetative frond or turion) are easily distinguishable, the switch is rapid (within 4 d), and the liquid culture, small size, and rapid growth of *S. polyrrhiza* presents many advantages for biochemical and physiological analyses.

Although a vast number of effects of hormones on plant development and physiology have been reported, the significance of these effects in vivo has generally been more difficult to interpret (Trewavas and Jones, 1991). In particular, many effects are seen only after the exogenous supply of relatively high concentrations of hormone, concentrations that the elicited tissue might never attain under normal growth conditions. We have addressed this problem in *S. polyrrhiza* by measuring the levels of ABA in tissue both during normal growth and after the induction of turions by ABA.

The analysis of ABA levels in a tissue presents a number of technical problems, in particular as to the specificity and accuracy of the data obtained (Neill and Horgan, 1987). We analyzed ABA levels by two independent methods. First, we used an ELISA, which allowed the rapid assay of ABA levels in a relatively large number of samples after only a limited number of purification steps. Second, we used an HPLC-linked GC-ECD method, which provided an assessment of the accuracy of the data obtained using the ELISA and also allowed the assay of both enantiomers of ABA. Both methods led to calculated values of ABA_i during (+)-ABA-induced turion formation in the range of 717 to 3923 pmol g^{-1}. At lower values of ABA_i (i.e. in control plants not producing turions) there was a tendency for the GC-ECD method to yield higher estimates of ABA_i relative to the ELISA. This was probably due to the limits of sensitivity of the two methods, compounded by the use of relatively high amounts of labeled tracer in the HPLC/GC-ECD analysis (required for estimation of recoveries) necessitated by the greater losses involved in the increased number of purification steps used. The general agreement in the values measured using the two independent methods for the analysis of ABA levels vouches for the accuracy of the data obtained.

As a result of this careful analysis of ABA levels in *S. polyrrhiza*, it is evident that during turion induction by the threshold concentration of exogenous ABA (250 nM) the level of ABA_i in control tissue increases from 75 pmol g^{-1} fresh weight (equivalent to approximately 75 nM) to approximately 1000 nM. This represents a 13-fold increase in ABA_i. It is possible to estimate the concentration of ABA present in the different cellular compartments both before and during turion induction by exogenous ABA (Cowan et al., 1982; Fleming et al., 1991) using reported values of the relative compartmental cellular volumes in *S. polyrrhiza* (cell wall, 5%; cytoplasm, 27%; vacuole, 68%; Smart and Trewavas, 1983b), standard estimates of cytosolic pH (7.4) and vacuolar pH (5.9) (Roberts et al., 1982), and the measured levels of tissue ABA reported here (control tissue 75 nM, induced tissue 1000 nM). Such calculations provide an estimate of ABA concentration in the cell wall of 20 nM, in the cytoplasm of 240 nM, and in the vacuole of 10 nM in control tissue. When the total tissue ABA concentration reaches 1000 nM, the cell wall ABA concentration increases to 270 nM, the cytoplasmic concentration increases to 3330 nM, and the vacuolar concentration increases to 120 nM. Indeed, in control cultures with a measured total concentration of 75 nM ABA in the fronds we measured a medium concentration of 15 to 25 nM ABA. Likewise, supplying fronds with an external concentration of 250 nM ABA we measured a total tissue concentration of 1010 nM. These estimates suggest that in our experiments the exogenous ABA was equilibrating within the tissue as predicted by mass action flow and provide an indication of the level of ABA required to be achieved in a cellular compartment if ABA is to act in that compartment by reaching a threshold concentration.

Our data suggest that a 13-fold increase in ABA level within a tissue would be sufficient to provide a turion-inducing concentration of ABA. We are at present analyzing the level of ABA attained in plants undergoing turion formation via signals other than ABA (e.g. cold, nitrate starvation), but a survey of the literature available indicates that a 13-fold stimulation of ABA level in a tissue is likely to be readily achievable (Walton, 1980; Harris et al., 1988; Capell and Dörffling, 1989; Goliber and Feldman, 1989; Perata et al., 1990; Singh and Browning, 1991; Parry et al., 1992).

It should be stressed here that, although our data suggest that turion induction by exogenous ABA is generally associated with an increase in ABA_i, this does not mean that in the in vivo situation an increase in ABA concentration is a priori required for turion formation. It is readily conceivable that, if ABA is involved in the mechanism by which environmental effectors induce turions, it might act via an altered signal transduction sensitivity mechanism rather than by an altered level of ABA itself (Trewavas and Jones, 1991). What our data provide is, first, evidence that the exogenous ABA concentration used to trigger the system is within the physiological range, i.e. that the level of ABA associated with turion formation is likely to be achievable in the tissue via endogenous synthesis. Second, they provide an estimate of the change in endogenous concentration required if environmental triggers are to act via a change in ABA level. Our ongoing analysis of ABA levels in plants forming turions in response to environmental effectors of turion formation will allow us to assay whether an increase in ABA concentration is indeed required for the transduction of these signals into the observed plant morphogenic response.

Both Enantiomers of ABA Can Induce Turion Formation

Although only the (+)-enantiomer of ABA has ever been identified in plant tissues, a number of ABA responses have been found to be inducible by both (+)- and (−)-enantiomers, and, indeed, a classification of ABA responses

into rapidly reversible or long-term developmental categories according to their (+) or (−) sensitivity has been proposed (Milborrow, 1980). Our results indicate that turions can be induced equally by both enantiomers of ABA, thus fitting into the correlation of developmental responses being sensitive to both enantiomers (Sondheimer et al., 1971; Walton, 1983; Walker-Simmons et al., 1992), although some exceptions have been reported (Churchill et al., 1992). However, our analysis of ABA levels in the tissue following treatment with (+)- or (−)-ABA indicates that, although both are equally effective in inducing turions, the subsequent tissue accumulation of ABA is quite different. In particular, treatment with (−)-ABA leads to a much higher accumulation of ABA in the tissue compared with treatment with (+)-ABA. Since it has been shown previously that incubation with (−)-ABA does not lead to an accumulation of (+)-ABA (Dunstan et al., 1992; Walker-Simmons et al., 1992), we conclude that the accumulation is due to the (−)-ABA enantiomer. Our data are substantiated by those of previous investigators, which also indicated that (−)-ABA is less readily metabolizable than (+)-ABA (Mertens et al., 1982; Vaughan and Milborrow, 1984; Abrams et al., 1989; Dunstan et al., 1992; Balsevich et al., 1994). Moreover, the apparently different accumulation of the enantiomers, yet similar efficacy, in turion induction has a significant implication regarding the site of action of ABA, as discussed below.

Site of ABA Perception Linked to Turion Formation

As a weak acid, ABA could theoretically interact with a receptor located either at the plasmalemma (outward-facing signal perception) or, after diffusion across the membrane, with a cytosol-located receptor (internal signal perception). The actual site of ABA perception remains unproven, although recently there has been much debate concerning this subject (Allan and Trewavas, 1994; Assmann, 1994). For example, investigations on guard cell opening and barley aleurone α-amylase synthesis have indicated an outward facing site of ABA perception (Anderson et al., 1994; Gilroy and Jones, 1994), whereas other works examining guard cell closure (Allan et al., 1994) and both guard cell closure and opening (Schwartz et al., 1994) have suggested an internal site of ABA perception.

The results presented in this paper indicate that, following treatment with ABA, there is an increase in the ABA_i level associated with turion formation. This would be consistent with an internal site of ABA perception. However, this correlation is not absolute, since tissue can occasionally attain turion-associated levels of ABA_i without observable turion formation. Moreover, our analysis of (+)- and (−)-ABA enantiomers indicates that, although they are similarly effective in eliciting turion formation, they lead to quite different levels of accumulation of ABA, suggesting that there is no simple internal trigger level of ABA required for turion formation. Furthermore, the results from our analysis of the effect of pH on ABA's induction of turions indicate that at high external pH (at which exogenously supplied ABA might be expected to be virtually membrane impermeable) ABA can still induce turions, albeit with a higher threshold. Decreasing the pH increases the sensitivity to ABA, resulting in a lower threshold concentration and a broader dose-response curve. However, calculation of the concentration of the membrane-permeable ABAH form at the different ABA concentrations and pHs examined revealed no correlation between ABAH concentration and turion formation.

Calculations of ABA distribution within a tissue based on simple anion trapping should be treated with some caution. For example, recent data suggest that the theoretical limits on ABA diffusion by pH may not always be reflected by the experimental situation (Daeter and Hartung, 1993), ABA distribution may be affected by specific transporters (Fleming et al., 1991), and the ABA-responsive tissue may represent only a fraction of the total plant used for quantitative ABA analysis. All of these factors tend to confuse a simple prediction of ABA levels and distribution within a tissue. Nevertheless, our results suggest that turion formation is not linked to the calculated uptake of ABA across the plasmalemma, although conclusive evidence would require, for example, the use of membrane-impermeable analogs of ABA or microinjection experiments.

In contrast to the potentially confusing interpretation of data on ABA_i levels and distribution, we consistently observe a very tight correlation between the exogenous concentration of ABA and turion induction. Moreover, although (+)- and (−)-ABA are equally effective in inducing turions at equivalent external concentrations, they accumulate to distinctly different levels within the tissue. These values do not suggest any simple trigger level of ABA_i for turion formation. When we consider all of these data together, the simplest interpretation is that ABA acts at an outward-facing, plasmalemma-localized receptor to initiate the signal transduction cascade that results in the coordinated cellular response involved in turion formation.

In conclusion, via a careful analysis of ABA levels during turion formation and of the effect of different enantiomers and growth conditions on the induction of this response by ABA, we have shown that ABA could play a physiological role in this process, and moreover, our data are consistent with the hypothesis that ABA initiates turion formation via interaction with a plasmalemma-associated receptor function. Our future experiments will be directed toward revealing whether ABA represents a unique regulatory pathway in this process or whether it can be circumvented by other signal transduction pathways and toward a dissection of the molecular events associated with turion induction via ABA.

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