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Auxin-binding Protein 1 Does Not Bind Auxin within the Endoplasmic Reticulum Despite This Being the Predominant Subcellular Location for This Hormone Receptor*

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Auxin-binding protein 1 (ABP1) is a unique hormone receptor because it resides primarily in the lumen of the endoplasmic reticulum (ER); however, two lines of evidence presented here suggest that ABP1 does not bind auxin within the endoplasmic reticulum, despite its predominant location there. First, ABP1 cannot be photolabeled in intact cells that have accumulated the auxin and photolabeling reagent 5-[7-^{3}H]azidoindole-3-acetic acid, indicating either that auxin is excluded from the ER and is not available for photolabeling to ABP1 or that binding conditions within the ER lumen are insufficient for photolabeling. Second, at the pH of the ER lumen, auxin binding to ABP1 is not detectable. The pH estimate of the ER lumen is based on an indirect assay, which indicates that the pH is closer to pH 7 than to the binding optimum of pH 5.5. These results indicate that ABP1 does not bind auxin within the ER and point to a site of action that is post-ER. The effect of auxin on its trafficking from the ER was tested in an animal expression system. ABP1 expressed at high levels in COS7 cells is efficiently retained in the ER lumen and is not secreted even in the presence of 190 μM indole-3-acetic acid, an auxin concentration that is 40 times above the K_d for indole-3-acetic acid binding to ABP1.

Hertel *et al.* (1972) reported auxin-binding in microsomes isolated from corn coleoptile cells and later designated this activity Site I. Several groups (Löbler and Klämbt, 1985; Shimomura *et al.*, 1986; Napier *et al.*, 1988) purified the protein responsible for this Site I activity (*cf*. Table I in Jones (1994)), and it has been shown directly that this protein binds auxin (Jones and Venis, 1989).

Several lines of evidence indicate that ABP1[1] in maize is an auxin receptor that acts at the plasma membrane. First, among a series of 45 auxins or similar compounds where binding affinity and growth induction was compared, there is a correlation between K_d and pC_{50}, except with some of the substituted phenoxypropionic acids (Ray *et al.*, 1977). A molecular model based on these data, in conjunction with data on the identification of residues in the binding site, point out that auxin binding to ABP1 involves specific molecular interactions, as expected for a receptor (Edgerton *et al.*, 1994; Brown and Jones, 1994). Second, a synthetic peptide encoding the terminal 13 residues of ABP1 significantly modulate the ion current across the plasma membrane of *Vicia faba* guard cells (Thiel *et al.*, 1993), while synthetic peptides from other regions of ABP1 do not modulate current activity. This suggests that there is a specific interaction between this domain of ABP1 and a plasma membrane component. The behavior of this ABP1 peptide mimics part of the behavior of auxin in the *V. faba* protoplast (Blatt and Thiel, 1994). Third, antisera directed against ABP1 blocks auxin-induced polarization of the plasma membrane on tobacco mesophyll protoplasts, indicating that ABP1 or an immunochemically similar protein mediates auxin-regulated ion movement (Barbier-Brygoo *et al.*, 1989, 1991; Rück *et al.*, 1993). Recently, one antibody to ABP1 also appears to block an auxin-modulated anion channel (Zimmerman *et al.*, 1994).

ABP1 has been shown to be located at the plasma membrane using immunocytochemisty in conjunction with electron (Jones and Herman, 1993) and silver-enhanced fluorescence (Deikmann *et al.*, 1995) microscopies. These data taken together indicate that ABP1 binds auxins in a specific and physiological meaningful manner at the plasma membrane to bring about a rapid hormone response.

An unusual feature of ABP1 is that it is localized to the lumen of the endoplasmic reticulum. Ray (1977) determined that the auxin-binding activity for ABP1 comigrates with the ER marker cytochrome *c* reductase during isopynic centrifugation. Subsequently, others (Shimomura *et al.*, 1988; Jones *et al.*, 1989; Napier *et al.*, 1992) demonstrated that most of the microsomal pool of ABP1 comigrates with the ER marker. The localization of ABP1 to the ER is consistent with the presence of an ER-retention signal on ABP1 (Hesse *et al.*, 1989; Inohara *et al.*, 1989; Tillmann *et al.*, 1989) but seems to contradict the results that support a plasma membrane site of action. Jones and Herman (1993) investigated the location of ABP1 immunocytochemically in maize cells and found that ABP1 is located in the endomembrane system but not in any other organelle. Most importantly, some ABP1 was found at the plasma membrane and within the cell wall space providing an explanation of how an ER protein such as ABP1 could potentially have a site of action at the outer face of the plasma membranes of these target cells. Recently, Deikmann *et al.* (1995) used silver enhancement of immunofluorescence microscopy to visualize ABP1 and found ABP1 clustered at the outer surface of the plasma membrane.

An important question is where within or outside the cyto-

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[1] The abbreviations used are: ABP1, auxin-binding protein 1; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 5-[^{3}H]N_3IAA, tritiated 5-azidoindole-3-acetic acid; IAA, indole-3-acetic acid; BMS, Black Mexican Sweet; DMEM, Dulbecco's minimal essential medium; PAGE, polyacrylamide gel electrophoresis; NAA, naphthalene-1-acetic acid; PIPES, 1,4-piperazinediethane sulfonic acid.

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plasm does ABP1 bind auxin? The answer to this question will direct research to the cellular location of the site of action of ABP1, providing clues of its function. For example, an ER site of action suggests a molecular chaperone function, whereas a post-ER site of action suggests that ABP1 is involved in regulated secretion, *e.g.* of cell wall materials necessary for growth. Alternatively, others have proposed that ABP1 acts on the outer face of the plasma membrane (Barbier-Brygoo *et al.*, 1989; Thiel *et al.*, 1993).

Another important question is if auxin causes ABP1 to translocate from the ER. It seems possible that auxin binding causes a cellular redistribution of ABP1 to its site of action, analogous to other well documented cases of ligand-regulated translocation. We formulate this testable hypothesis from observations made by Napier and Venis (1990). They showed that a monoclonal antibody (designated MAC256) detected a ligand-induced conformational change in ABP1 that was subsequently mapped to or very near the carboxyl terminus (Napier *et al.*, 1992). A microtiter plate-based assay was developed to show ligand-dependent recognition of ABP1 by MAC256. Several auxins and structurally-similar compounds were tested for the ability to block recognition of MAC256 to ABP1, and there was a qualitative correlation between auxin activity, but not necessarily binding affinity, and inhibition of MAC256 recognition. Therefore, this raises a potential mechanism by which auxin regulates ABP1 trafficking. Specifically, auxin binds to ABP1 in the lumen of the ER and causes the KDEL retention signal to be masked, consequently allowing the passage of ABP1 to the plasma membrane, its proposed site of action.

Our hypotheses are specific and make certain testable predictions. 1) The conditions for auxin binding to ABP1 in the ER lumen are adequate, if not optimal. 2) Auxin is accessible to the ER lumen and to ABP1. 3) The structural information for auxin-regulated trafficking of ABP1 resides in the ABP1 sequence itself, therefore ABP1 should show auxin-regulated trafficking in a nonplant cell.

The first and second predictions are tested here by indirect measurements of the pH of and relative auxin concentration in the ER and by photolytic tagging of ER-localized ABP1 by 5-azidoindole-3-acetic acid (5-N_3IAA). Because ABP1 expressed in insect cells is native and active (Macdonald *et al.*, 1994), it should be possible to test the third prediction in a nonplant cell. COS7 cells were chosen for this because of the constituent expression of the T antigen enabling high level expression. Moreover, COS7 cells should lack any unique contribution that a plant cell may make in trafficking ABP1. Thus, the effect of auxin directly on ABP1 that causes its translocation from the ER *versus* some indirect effect occurring in plant cells should be revealed using these animal cells.

MATERIALS AND METHODS

Chemicals, Cells, and Tissue—Hybrid maize (*Zea mays L.* hybrid B73 X Mo17 and J7710, Jacques Seed, caryopses were grown on wetted vermiculite or cotton for 3–4 days at 27 °C in darkness. The maize hybrid used to test the effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was WF9 X BR38 (Custom Farm Seeds, Decatur, IL). Black Mexican Sweet (BMS) maize cells were cultured as described in Jones and Herman (1993). COS7 cells were obtained from the Lineberger Cancer Center, University of North Carolina. COS7 cells were maintained on Dulbecco's minimal essential medium (DMEM) supplemented with 4500 mg/liter L-glutamine and 10% fetal calf serum (DMEM-10). [^{3}H]IAA (940 GBq/mmol) and [^{3}H]NAA (651 GBq/mmol) were purchased from Amersham Corp., and 5-azido-[^{3}H]IAA (740 GBq/mmol) was synthesized as described in Melhado *et al.* (1982). Most other chemicals were purchased from Sigma.

Construction of pHTa—The cDNA encoding full-length ABP1 was amplified from pUC800 (Tillmann *et al.*, 1989) using primers containing *Bam*HI restriction sites and subcloned in both directions into pGEMex1 (Promega). The orientation and sequence was verified by DNA sequencing. *Bam*HI inserts were cloned into pSG5 (Stratagene), and the orientation was verified by restriction mapping. pSG5 contains the SV40 early promoter followed by a β globulin intron to elevate expression. The SV40 promoter is under the control of the T antigen, which is constitutively expressed in COS7 cells.

Transfection of COS7 Cells—A green monkey kidney cell line (COS7) was transfected by the DEAE-dextran method as described in Ausubel *et al.* (1990) with slight modification. Briefly, COS7 cells were grown to approximately 50% confluency in DMEM supplemented with 1% fetal calf serum. Cells were washed with phosphate-buffered saline, and fresh DMEM-1 was added. Plasmid (5 μg) with DEAE-dextran (10 mg/ml) thoroughly suspended in 3 ml of DMEM-1 was added dropwise to the cells and mixed by swirling the plates. After 4 h, the DNA·DEAE-dextran was aspirated off, and the cells were shocked with 10% Me_2SO in phosphate-buffered saline for 1 min. Fresh DMEM-1 was added, and the cells were grown for a maximum of 72 h at 37 °C in 5% CO_2. IAA was added immediately after transfection. The medium and cells were collected at different times for immunoblot analysis.

Fluorescence Microscopy—Transfected cells were grown on a glass coverslip. Cells were washed in phosphate-buffered saline and fixed with 1% formaldehyde in 0.1 M PIPES, 10 mM EGTA, 20 mM $MgSO_4$, 1% Nonidet P-40, pH 7.45, for 10 min. Cells were probed with rabbit anti-ABP1 serum (NC04, 1:1,000) overnight, washed in phosphate-buffered saline, and incubated with goat anti-rabbit Ig conjugated to rhodamine for 2 h. Washed cells were then viewed using a Nikon Optiphot with epifluoresence.

Radioanalysis of IAA—Approximately 1 μCi of [^{3}H]IAA was added to confluent COS7 cells grown either in T25 flasks (10-ml cultures) or on 12-well plates (1.6-ml cultures). After 24 h, the media were collected, and the cells were washed in an equivalent volume of DMEM and extracted twice with 0.5 ml of methanol. Radioactivity in the media and methanolic extracts was determined by liquid scintillation counting. The methanolic extract was reduced to near dryness by evaporation under streaming nitrogen and analyzed by thin-layer chromatography, silica, ethyl acetate/isopropyl alcohol/concentrated ammonia (45:35:20).

Photoaffinity Labeling ABP1 in Vivo and *in Vitro*—Auxin binding *in vivo* was performed as described in Jones (1990) with the following modifications. The coleoptile was removed from the shoot with special care to avoid any leaf tissue. Coleoptiles (8 g) were cut into 0.5-cm lengths and incubated for 3 h in buffer (5 mM sodium citrate, pH 5.5) and then transferred to buffer containing 5 μM 5-[^{3}H]N_3IAA in the dark for 3 h. Coleoptile tissue was rinsed with water and irradiated with UV as described in Jones (1990) except that the two UV sources were mounted closer (2 cm) to the tissue. It was not possible to determine the exact amount of energy from the UV sources mounted so closely, but it is well over 10 milliwatts/cm^2. Microsomes were prepared from coleoptiles (8 g) as described in Jones *et al.* (1984), except that the volume of the resuspended microsomal pellet was adjusted so that it had an absorbance at 254 nm equal to that of the tissue. Microsomes were incubated in 5 μM 5-[^{3}H]N_3IAA for 30 min at 4 °C and irradiated simultaneously with the tissue. ABP1 was enriched by *n*-butyl alcohol extraction of the microsomes followed by ion-exchange chromatography (Q-Sepharose, Pharmacia Biotech Inc.). Protein concentrations were determined using the method described by Bradford (1976).

Auxin-binding Assays, SDS-PAGE, and Immunoblot Analysis—The auxin-binding assays using crude extracts of maize coleoptile were performed exactly as described in Jones *et al.* (1984). SDS-PAGE and immunoblot analysis was performed as described in Jones and Herman (1993).

Radioactive Auxin Distribution in Microsomes—Coleoptile tips 10–15 mm in length were collected, and 1-g samples were treated in 2 ml of 10 mM phosphate buffer, pH 6.0, plus the indicated radiolabeled auxin for 4 h. Similar treatments were done with BMS cultured corn cells (Jones and Herman, 1993). 0.2 g of loosely packed BMS cells washed free of 2,4-dichlorophenoxyacetic acid were used per 2 ml of 2,4-dichlorophenoxyacetic acid-free medium plus radiolabeled auxins as indicated for 2–4 h. Samples were homogenized in binding buffer I (10 mM sodium citrate, 0.5 mM $MgSO_4$, 250 mM sucrose, pH 7.4) within a microcentrifuge tube fitted with a plastic pestle using and parallel samples were homogenized in binding buffer II (10 mM sodium citrate, 0.5 mM $MgSO_4$, 250 mM sucrose, pH 5.5). Homogenates (1 ml) of each sample plus 1 additional ml used in the wash to assure complete transfer were centrifuged at 8,000 × *g* for 20 min. The supernatant (2.5 ml), designated supernatant 8K (*S8*), centrifuged at 80,000 × *g* for 30 min to provide fractions designated supernatants 80K (*S80*) and the microsomes (*M*). Radioactivity in each sample was determined by liquid scintillation counting. The hydrated weights of microsomes were measured before resuspension in 0.1 ml of the respective buffer for measuring the radioactivities. Parallel samples without incubation in radio-

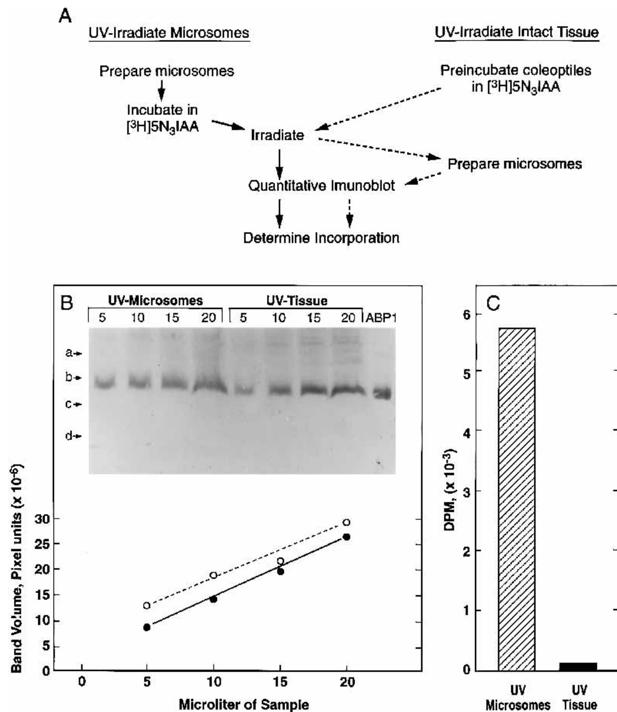

FIG. 1. **Maize ABP1 does not bind 5-[^{3}H]N$_3$IAA *in vivo*.** *A*, experimental scheme. Coleoptile tissue in 0.5-mm sections was incubated in 5-[^{3}H]N$_3$IAA for 3 h and irradiated with intense UV light to photolabel ABP1 with 5-[^{3}H]N$_3$IAA. Incorporation of 5-[^{3}H]N$_3$IAA was compared with the maximal incorporation possible using isolated microsomes. *B*, ABP1 photolabeled in microsomes (*UV-Microsomes*) or in coleoptile (*UV-Tissue*) was partially purified and subjected to immunoblot analysis. Increasing loads of each sample (shown as μl of sample) were compared to demonstrate that both treatments contain approximately equal amounts of ABP1. The blot was scanned, and the signal for each sample, expressed as pixel units, is shown to be linear with similar slopes. Molecular weight standards are indicated by letters: *a*, for ovalbumin; *b*, for carbonic anhydrase; *c*, for β lactoglobulin; and *d*, for lysozyme. Pure ABP1, not subjected to photoaffinity labeling, is shown. *C*, bands were excised from the blot and dissolved in methanol for liquid scintillation counting. Incorporation of the radioisotope for ABP1 photolabeled in microsomes (*hatched bar*) is compared with ABP1 photolabeled *in vivo* (*solid bar*). The amount of signal analyzed is from 80 μl of sample.

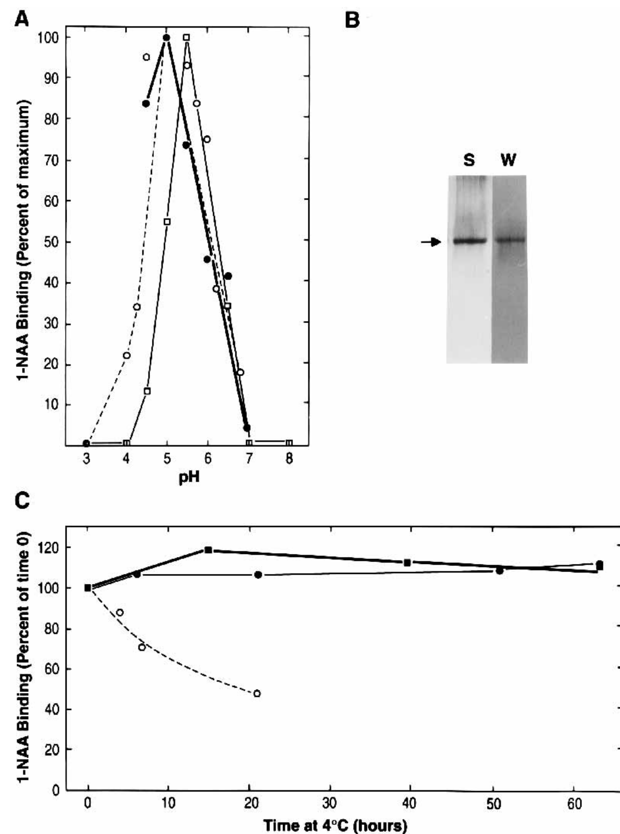

FIG. 2. **The pH of the endoplasmic reticulum is estimated to be near pH 7, which is far from optimal for auxin binding to ABP1.** *A*, the pH dependence for auxin binding to ABP1 (*boldface line*) was determined as described under "Materials and Methods." This data is compared with the data replotted from Löbler and Klämbt (1985) (*thin solid line*) and Shimomura *et al.* (1986) (*dashed line*). *B*, maize ABP1 was purified to homogeneity as described under "Materials and Methods." The ABP1 used in this study was subjected to SDS-PAGE and silver staining (*S*) and to immunoblot analysis (*W*). *C*, auxin binding in crude microsomal preparations of coleoptile tissue stored at 4 °C (*boldface solid line*, *solid square*) is compared with pure ABP1 stored at 4 °C either at pH 7 (*thin solid line*, *solid circle*) or pH 5.5 (*dashed line*, *open circle*). Auxin binding was performed at pH 5.5 as described under "Materials and Methods."

labeled auxin were homogenized in both binding buffers, supplied with radiolabeled auxins. All further procedures were the same as described above. The concentrations of radioactivity is expressed as disintegrations/min/μl of supernatant or mg of microsomes. The hydrated volume of 1 mg of microsomes is assume to approximate 1 μl.

RESULTS

ABP1 in Coleoptiles Is Not Photolabeled by 5-[^{3}H]Azidoindole-3-acetic Acid in Vivo—We addressed whether ABP1 in the ER lumen is able to bind auxin using the experimental scheme shown in Fig. 1. Coleoptile tissue, which was incubated in buffer to remove endogenous auxin, accumulated 5-[^{3}H]N$_3$IAA at an external concentration of 5 μM for 3 h in darkness. It has been previously shown that under these conditions that 5-[^{3}H]N$_3$IAA accumulates into the tissue severalfold over the external concentration and that this compound is transported through the tissue in a polar fashion at rates the same as for IAA, the endogenous hormone (Jones, 1990; Jones *et al.*, 1991). After incubation, the tissue was irradiated with intense UV to cross-link ABP1 with the photoaffinity auxin, 5-[^{3}H]N$_3$IAA. The amount of incorporation of tritium was determined and compared with the maximum amount of photolabeled ABP1 in microsomes having the same amount of UV absorbance. Background labeling occurred in both treatments nearly equally (data not shown), indicating that 5-[^{3}H]N$_3$IAA entered cells and that the UV irradiance was sufficient for *in vivo* photoactivation. Fig. 1 shows that little, if any, ABP1 was photolabeled *in vivo*. In contrast, isolated microsomes that have been adjusted by buffer to optimal binding conditions contain ABP1 that was efficiently photolabeled by 5-[^{3}H]N$_3$IAA. This indicates that 1) *in vivo* there is very little auxin in proximity to the major pool of ABP1 and/or 2) the conditions for auxin binding to ABP1 in the ER lumen are not optimal.

The pH of the ER Lumen Is Not Optimal for Auxin Binding to ABP1—It has been estimated that the pH of the ER lumen is approximately 7 (discussed below). At this pH, there is no detectable auxin binding to ABP1, as shown in Fig. 2*A*. We estimate using the following method that the pH of the lumen *in vivo* is closer to pH 7 than pH 5.5 by taking advantage of the fact that ABP1 half-life (binding activity) is pH-dependent (Shimomura *et al.*, 1986). At pH 5.5, the half-life for auxin binding to ABP1 is considerably shorter than at pH 7, 4 °C (Fig. 2) (Shimomura *et al.*, 1986). We show the decay of ABP1 activity *in vivo* and compared this with the decay of pure ABP1 activity at pH 5.5 and 7.0 in solution. Shoots of maize seedlings were stored in the dark at 4 °C, and the auxin binding capacity in the coleoptile was determined over 4 days. Fig. 2 illustrates that the auxin binding activity of ABP1 is unchanged over time during storage. Highly pure ABP1 (shown in Fig. 2*B*) was

stored at pH 5.5 or 7.0 and measured for auxin binding over time. The auxin-binding activity of pure ABP1 stored at pH 7 (4 °C) was stable, whereas ABP1 stored at pH 5.5 decayed rapidly (Fig. 2*C*). Considering the stability of ABP1 *in vivo* and the cellular localization of ABP1, we conclude that the pH value of the ER lumen is near neutrality.

Isolated Microsomes Do Not Contain a High Concentration of Auxin—Based on the estimated neutral pH in the ER and the narrow optimum of pH 5.5 for auxin binding to ABP1, we reasoned that the occupancy by auxin of ABP1 in the ER should be low. We therefore attempted to determine if a plant cell compensates for the effect of neutral pH by a mechanism to make the amount of auxin well in excess over ABP1 in the ER lumen. Conceptually, this is the mechanism for driving occupancy of the acetylcholine receptor by acetylcholine, where a low affinity binding is compensated by a ligand concentration well in excess of the receptor concentration.

Excised maize coleoptiles and BMS maize cells were incubated with [^{3}H]IAA or [^{3}H]NAA for 4 h in phosphate buffer, pH 6.0, and then after homogenization either with pH 5.5 or pH 7.0 buffers, and the amount of radioactivity in the supernatant and the microsome was determined for each (Fig. 3). In addition, an experiment was performed where the radiotracer was added during homogenization of the tissue. The pH of the buffer had no effect on the distribution of auxin between the supernatant and the microsomes. Also, the same distribution of auxin was obtained when the radiotracer was added during grinding. These data suggest that auxin is in equilibrium between the cytosol and the ER lumen, that there is no facilitated uptake, and that the concentration of auxin in the cytosol is similar to the concentration within the ER lumen.

We also determined that there is no significant pH gradient across the isolated microsomal membrane. Auxin binding in microsomes was measured in the presence and absence of the protonophore, FCCP. Fig. 4 shows that the total amount of auxin binding and auxin-binding affinity is not affected by FCCP, although the background level of binding is 10% higher in the control samples.

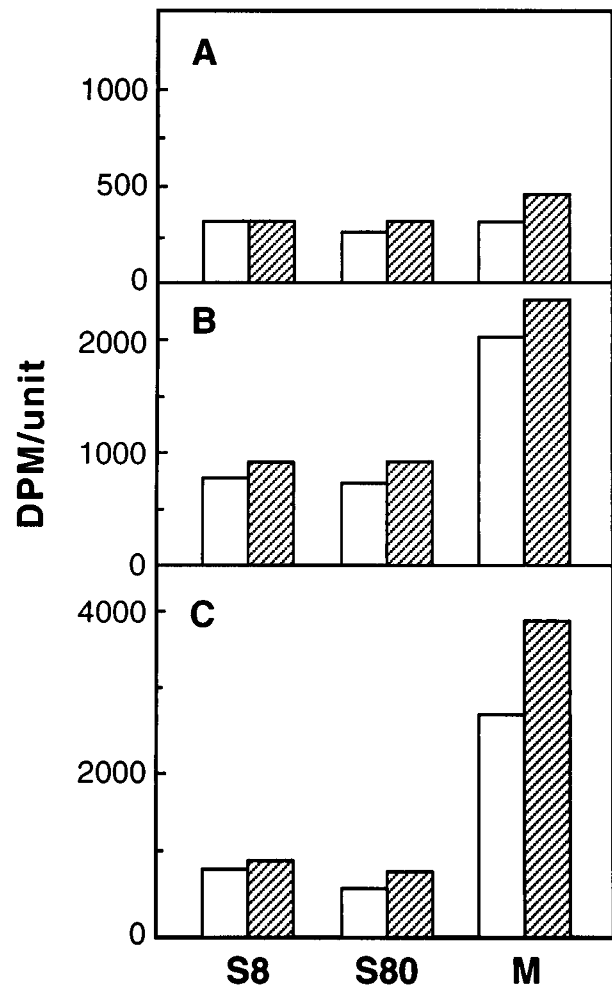

FIG. 3. **Distribution of radioactive auxins in the soluble and microsomal compartments of maize coleoptile cells determined after cell homogenization.** Coleoptiles were incubated in [^{3}H]IAA (*panel A*) or [^{3}H]NAA (*panel B*) for 4 h and then homogenized either in a pH 5.5 buffer (*open bars*) or a pH 7.0 buffer (*solid bars*) and fractionated by differential centrifugation as described under "Materials and Methods." In *panel C*, tissue was homogenized in the presence of [^{3}H]NAA, and the cell contents were fractionated as above. *S8* and *S80* represent the supernatants from centrifugations at 8000 and 80,000 × *g*. *M* represents the microsomal pellet from the centrifugation at 80,000 × *g*. Radioactivity in each of these fractions is represented as disintegrations/min/μl for the supernatants (*S8* and *S80*) or as disintegrations/min/mg of microsomes (*M*). During the incubation period, coleoptile cells took up almost half of the exogenous [^{3}H]IAA and two-thirds of the exogenous [^{3}H]NAA. The standard error of the mean for the disintegrations/min/unit is 10% or less. The same results were obtained using BMS cells.

ABP1 Expressed in COS7 Cells Is Not Secreted in the Presence of Auxin—An ABP1 cDNA under the control of the SV40 early promoter was used to transfect COS7 cells using the DEAE-dextran method for transient expression (Ausubel *et al.*, 1990). The level of expression was followed for 2 days by immunoblot analysis and is shown in Fig. 5. Adding auxin at a high concentration in these cells had no effect on the expression level of ABP1 (Fig. 5) or on the growth rate and cell morphology (data not shown). The highest level of expression occurred by 36 h and decreased as the cells became over-confluent. As seen in Fig. 6, most of the expressed ABP1 had an identical subunit molecular mass as maize ABP1, suggesting that ABP1 is correctly processed in COS7 cells. The small amount of a 24-kDa protein is also observed in Fig. 6 and may be due to partial glycolytic processing of ABP1.

ABP1 was not detected in the medium (Fig. 6). The addition of 190 μM IAA, added either once after transfection or twice over the time course of the experiment, did not induce ABP1 secretion. The ABP1 standard shown in Fig. 6 represents a signal that is less than 1% of the signal shown for ABP1 present in COS7 cell extracts. Since the same portion of cell extract is compared with medium, this indicates that the steady state amount of ABP1 in the medium over 2 days is well below 1% of the total cellular ABP1 population.

The distribution of ABP1 in COS7 cells was examined by immunofluorescent microscopy. ABP1 staining distributed in a typical ER pattern (Fig. 6). Staining of the periphery of the nuclear envelope in addition to punctate and elongated structures suggests ABP1 localization in cisternal and tubular ER and possibly cis Golgi. The preimmune controls (Fig. 6) indicate that the fluorescent signal is solely due to ABP1.

IAA was shown to enter COS cells by growing cells in the presence of [^{3}H]IAA and quantitating the uptake of IAA into cells by liquid scintillation. Using packed cell volume, the internal IAA concentration (400,000 dpm/ml) was calculated and found to be approximately equal to the external IAA concentration (335,000 dpm/ml), indicating that IAA is not excluded from COS7 cells.

IAA is stable in COS7 cells. The stability of IAA was demonstrated by adding [^{3}H]IAA to confluent cultures and after 24-h methanol extracts of the cells were examined by thin-layer chrmatography as shown in Fig. 7. IAA extracted from COS7 cells had the same radiopurity as authentic [^{3}H]IAA.

The lack of ABP1 in the medium (Fig. 6) suggests the follow-

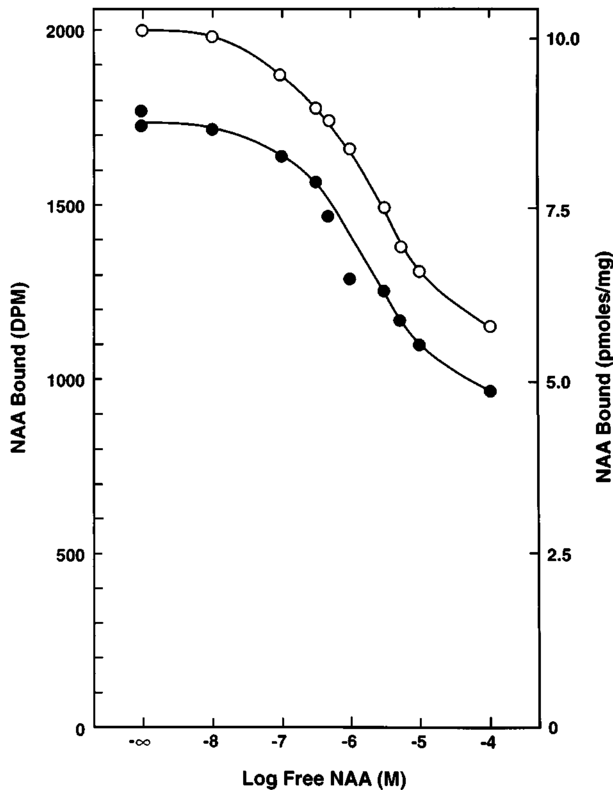

FIG. 4. **Isolated microsomes do not have a pH differential as indicated by the lack of an effect of the protonophore, FCCP, on auxin binding.** Microsomes were prepared from coleoptiles and analyzed for competitive auxin binding in the presence (*solid circles*) and absence (*open circles*) of FCCP.

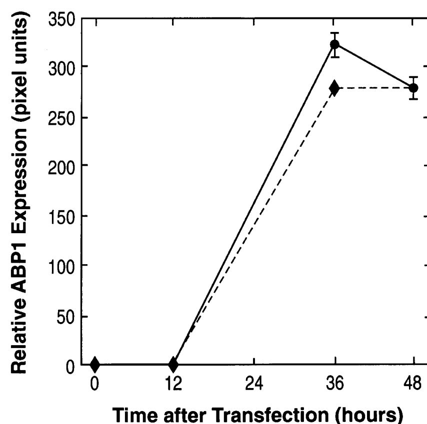

FIG. 5. **Time course for expression of maize ABP1 in COS7 cells.** COS7 cells were transfected with pHTa as described under "Materials and Methods" and grown on multiple plates. At the times indicated, cells were harvested from a single plate and extracted in SDS-PAGE buffer. 2% of the cells or the medium was loaded in each lane. One series of plates included 190 μM IAA added at the initial plating. Extracts from an equal number of cells from each time point were subjected to SDS-PAGE (12%) and immunoblot analysis. Blots were probed with antiABP1 (NC04, 1:10,000), and the ABP1 signals were analyzed using a Molecular Dynamics image analyzer. The volume of each band was determined and the relative ABP1 expression in the presence (*diamond*) and absence (*circles*) of IAA is shown as pixel units.

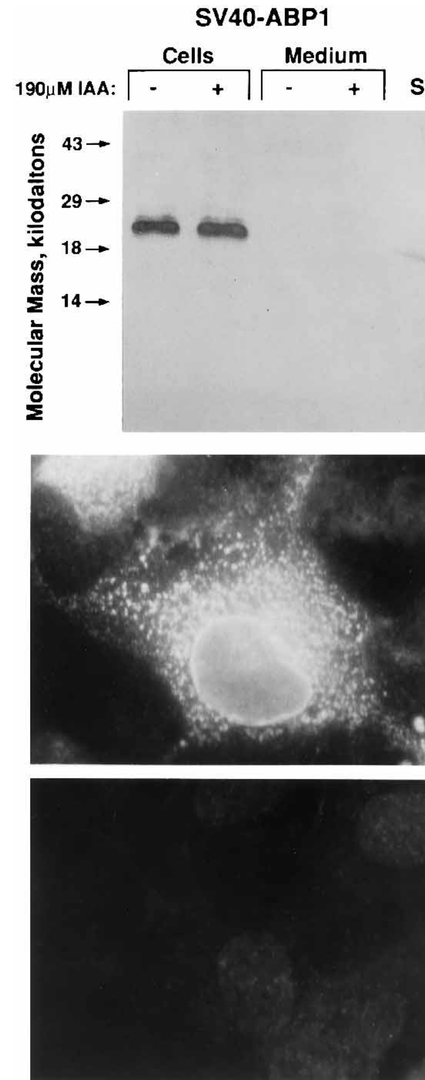

FIG. 6. **ABP1 is expressed at high levels in COS7 cells and is not detectably secreted.** COS7 cells were transfected with pHTa as described under "Materials and Methods" and plated in the presence (+) or absence (−) of 190 μM IAA and grown for 48 h, at which time the cells and media where collected and subjected to SDS-PAGE and immunoblot analysis (*top panel*). An amount equivalent to 2% of cells or media was loaded per lane. Blots were probed with antiABP serum (NC04, 1:10,000). Purified maize ABP1 was loaded so that the signal was approximately 1% of the signal for ABP1 in COS7 cells. Cells were also fixed and probed with antiABP1 serum (NC04, 1:1,000; *middle panel*) or the preimune serum (*bottom panel*).

ing three possibilities. 1) COS7 cells efficiently retain ABP1 even in the presence of auxin. 2) ABP1 is secreted but rapidly degraded outside the cells, or 3) ABP1 is secreted but rapidly taken up. To distinguish between these possibilities, ABP1 purified from maize seedlings was added to confluent cultures of COS7 cells to determine its stability. This stage of cell growth was chosen because it is the time at which there is maximum expression of ABP1 in transfected COS7 cells (Fig. 5) and the most likely time when extracellular proteolysis might occur. The medium was examined for the amount of ABP1 at several times and compared with ABP1 incubated in DMEM-10 alone (no cell controls) at each time point. Fig. 8 illustrates that ABP1 is stable in COS7 medium and that the addition of IAA does not affect this stability. Since the added ABP1 is stable in the presence of COS7 cells (Fig. 8) and the transiently expressed ABP1 is not detectable in the medium (Fig. 6), we conclude that ABP1 is not secreted in COS7 cells.

DISCUSSION

This work addresses the question of whether ABP1 binds auxin in the ER, and whether this binding causes a redistribution of ABP1 from the ER to post-ER compartments. These ideas have been topics of speculation since auxin binding (Site I) was discovered within the ER (Hertel *et al.*, 1972; Ray *et al.*,

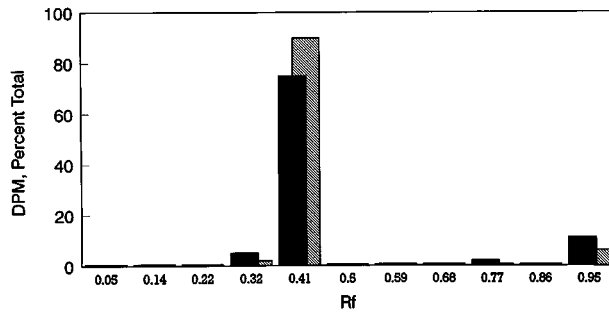

FIG. 7. **[³H]IAA is not metabolized by COS7 cells.** [³H]IAA was added to plates of confluent COS7 cells and to plates containing DMEM-10 medium alone. 24 h later, the radioactivity in the cells was determined by extracting washed cells with MeOH and analyzed by thin-layer chromatography as described under "Materials and Methods." Extracted radioactivity, *stippled bars*; pure [³H]IAA, *solid bars*.

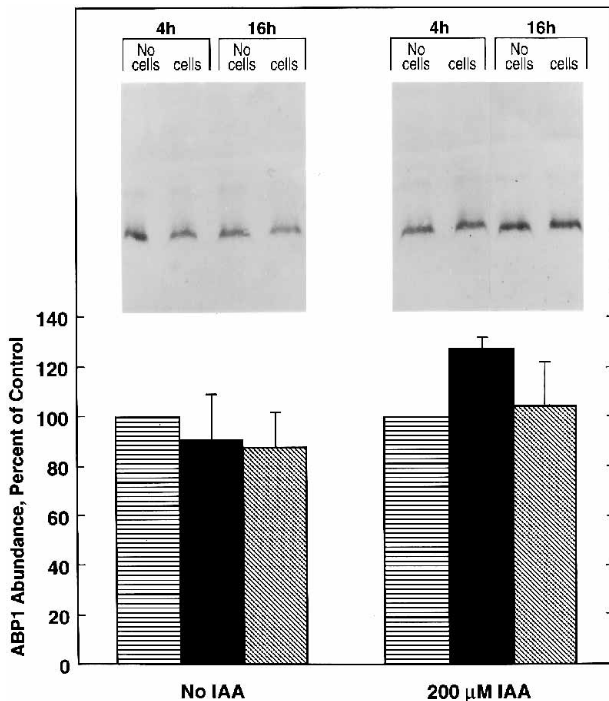

FIG. 8. **Maize ABP1 is not degraded in the medium of COS7 cells.** Pure maize ABP1 was added to the medium of COS7 cells, grown to confluency on 12-well plates, or added to plates without cells (*No cells*). In addition, IAA was either present (200 μM IAA) or not. After 4 and 16 h, the medium was sampled and subjected to SDS-PAGE and immunoblot analysis. Blots were probed with antiABP1 serum (NC04, 1:10,000), and the volume of the ABP1 bands was determined by image analysis. A typical blot is shown in the *upper part* of the figure. The average relative signal based on three blots, with multiple lanes of samples, each scanned twice, is shown in the *lower part* of the figure. The ABP1 signal in the *No cells* control (*cross-hatched bars*) is set as 100% and the amount of ABP1 remaining in the media from cells at 4 h (*solid bars*) and 16 h (*stippled bars*) is expressed as a percent of the control. The error is expressed as S.E.

1977). For example, "the bucket brigade" model was put forth by Ray (1977) to explain a possible mode of auxin-induced proton excretion. In this model, auxin binds to its receptor in the ER and somehow cause an increase in the exocytosis of acidic vesicles carrying cell wall materials. Cross (1991) has proposed that ABP1 cycles between the ER and the plasma membrane and that elevated auxin accelerates this cycling. In both models, it is proposed that the response of auxin binding to its receptor in the ER stimulates exocytosis of materials/enzymes used to expand the cell walls.

Auxin binding to ABP1 in the ER requires that auxin be present in this compartment and that the binding conditions are near optimal. Specifically, because auxin binding is strictly dependent on pH, an ER pH near 5.5 is one requirement for 100% occupancy. Contrary to this, indirect evidence, which is discussed below, support a neutral pH, yet, at this pH, auxin binding to ABP1 does not occur or does so below detection. An argument dealing with this dilemma (Shimomura *et al.*, 1986) has been that the compromise between a low pH for binding optimum, and a neutral pH/oxidative redox state for proper folding (Hwang *et al.*, 1992) has evolved as a part of ABP1 mode of action. A counter argument is simply that ABP1 does not bind auxin in the ER lumen but rather in a post-ER compartment where the pH is at or closer to the optimum for binding. As discussed, patch clamp experiments reveal control of ion channels by ABP1 on the outer face of the plasma membrane (summarized in Goldsmith (1993)). Because the plasma membrane/cell wall space has a pH of 5.5–6.0 (Cleland, 1976; Hoffman *et al.*, 1992; Jacobs and Ray, 1976), this proposed site of auxin perception by ABP1 remains plausible.

There is no method currently available to directly measure ER lumenal pH; however, based on indirect measurements and predictions about the ER microenvironment based upon the characteristics of several ER proteins, it is generally accepted that the ER pH is approximately 7. A neutral pH value has been the basis for the structure of some ER proteins and mechanisms of their function (*e.g.* Wilson *et al.*, 1993; Yoo and Lewis, 1992). The evidence for the ER pH is based on a variety of approaches. For example, 2,4-(dinitroanilino)-3′-amino-*N*-methylodipropylamine accumulates into acidic compartments but was not found in the ER lumen, suggesting that there is not a pH gradient between the ER and cytosol (Anderson and Pathak, 1985). Acidification of the ER to pH 5.8 disrupts the trafficking of secreted proteins such as lysozyme (Pilarsky and Koch-Brandt, 1992), which is consistent with the recent observation that inhibition of a H^+-ATPase disrupts protein trafficking in the post-ER compartments but not ER to Golgi movement (Yilla *et al.*, 1993). The pH dependence for activity of several ER proteins has also indicated a neutral or near neutral ER lumenal pH. For example, the ER isoform of ethanolamine-phosphate cytidylyltransferase of castor bean endosperm has an optimum pH for activity between 6.5 and 8 (Wang and Moore, 1991). Bilirubin UDP-glucuronosyltransferase, an ER protein, has a pH optimum that is above 6.4 and has no activity at pH 6.0 (Ritter *et al.*, 1993).

Our estimate of ER pH is consistent with the above results, suggesting that the ER lumenal pH is near neutral. This finding suggests that the ER lumen is not the site of perception for auxin by ABP1. Alternative interpretations require assuming that ABP1 somehow remains stable in an acidic subcompartment of the ER. Therefore, it is more likely that post-ER compartments such as the trans Golgi or the outer surface of the plasma membrane, which have a pH that is optimal for auxin binding, is the site of auxin perception by ABP1. The short half-life of ABP1 expected for these cellular locations is consistent with a regulatory role for ABP1. If active receptor accumulates at the plasma membrane, the amount of auxin to obtain half-maximal occupancy becomes unreasonably high (Cheng and Prusoff, 1973). Furthermore, if the response of auxin at the plasma membrane (Thiel *et al.*, 1993) is not first order with respect to bound receptor complex but rather limited by a second effector as has been proposed (Klämbt, 1990; Barbier-Brygoo *et al.*, 1991), then it is necessary that the amount of active receptor be kept low. A short half-life for ABP1 at its site of action based on its instability at acidic pH may provide such a mechanism to prevent accumulation of active receptor.

In a variety of cases, ligand binding to its receptor causes a

redistribution of the complex receptor or binding protein (Picard and Yamamoto, 1987; Ronne *et al.*, 1983; Shreck *et al.*, 1991). The hypothesis that auxin binding causes a translocation of ABP1 from the ER to its post-ER site of action is attractive because it provides an immediate function of auxin and explains how a receptor carrying an ER retention signal could have an extracytoplasmic site of action. If this hypothesis is true, then the data from Napier and Venis (1990) based on purified ABP1 would suggest that the information for auxin-regulated redistribution resides within the structure of ABP1. This suggestion prompted us to test the hypothesis that auxin causes ABP1 translocation in a heterologous system where specific and unique plant trafficking components would be absent. An observed effect of auxin on ABP1 secretion would support this hypothesis; however, our results show that the expressed ABP1 remains within the ER of the COS7 cells even in the presence of auxin at a concentration 50 times above the K_d for auxin binding to ABP1. This work also indicates that the lower efficiency for ABP1 retention in plant cells relative to immunoglobulin binding protein and protein disulfide isomerase (Jones and Herman, 1993) may be due to a special component of the plant cell and not due to poor presentation of the KDEL sequence at the carboxyl terminus of ABP1 since ABP1 is efficiently retained in a nonplant cell.

While the above interpretation of our data is the simplest, we do not exclude other interpretations. For example, translocation of ABP1 to the cell surface is impaired at a certain step in COS7 cells due to an incompatibility of the cellular translocation systems between plant and animal cells. There may be multiple retention mechanisms in animal cells that preclude auxin-regulated translocation of ABP1, whereas this multiplicity may be absent in plant cells. While the concept of multiple retention mechanisms has been proposed, such as the "first line of defense" hypothesis of Rothman and Orci (1992), there is yet no evidence that retention of ER proteins in plant cells is substantially different than in animal cells.

The mechanism by which a small percentage of the ABP1 population is found at the plasma membrane and in the cell wall space is not known (Jones and Herman, 1992; Deikmann *et al.*, 1995). This small amount of extracytoplasmic ABP1 may be solely the result of an inefficient retention mechanism for ABP1 in plant but not animal cells. This unique property of ABP1 may have coevolved with (or selected for) the mechanism of ABP1 action at the plasma membrane. Alternatively, there may be a specific mechanism regulating ABP1 movement differently than other KDEL sequences in plant cells. To different degrees, all ER/Golgi proteins are expected to be found on the plasma membrane since retention and targeting is not 100% efficient, and in some cases small amounts of these proteins also have specific functions on the plasma membrane. For example, 5–10% of the mannose-phosphate receptor, a protein whose role in prelysozomes has clearly been established, is found on the plasma membrane where it serves to anchor acid hydrolases (Kornfeld, 1992).

An ER protein having a specific function in a post-ER compartment is not unique to ABP1. Animals cells have a soluble (39–44 kDa) protein containing an ER retention signal that interacts with three members of the low density lipoprotein receptor family (VLDP, gp330, and LRP receptors), which are located on the plasma membrane (Battey *et al.*, 1994; Kounnas *et al.*, 1992a, 1992b; Orlando *et al.*, 1992; Strickland *et al.*, 1991). This protein, designated RAP for receptor-associated protein, is found predominantly in the ER (Abbate *et al.*, 1993), but small amounts have been localized to the plasma membrane using radioidionation to tag cell surface proteins (Strickland *et al.*, 1991) and by immunoelectron microscopy (Pietromonaco *et al.*, 1990; Abbate *et al.*, 1993). Interaction of RAP with very low density lipoprotein, gp330, or LRP receptors inhibits uptake by these membrane receptors of serum ligands such as specific lipoproteins, proteases, protease/inhibitor complexes (Strickland *et al.*, 1994), and also the *Pseudomonas* exotoxin (Kounnas *et al.*, 1992b), which itself contains an ER retention signal (Chaudhary *et al.*, 1990). Little is known about how RAP translocates to the plasma membrane or its potential regulatory role at the plasma membrane.

By excluding the endoplasmic reticulum, these results narrow the cellular site of perception of auxin by ABP1. While current data are consistent with the view that ABP1 has a site of action at the plasma membrane (Goldsmith, 1993), these or previously published data do not exclude an intracellular post-ER site of action. Nor do they exclude a function within the ER that does not require auxin binding.

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REFERENCES

Abbate, M., Bachinsky, D., Zheng, G., Stamenkovic, I., McLaughlin, M., Niles, J. L., McCluskey, R. T., and Brown, D. (1993) *Eur. J. Cell Biol.* **61,** 139–149

Anderson, R. G. W., and Pathak, R. K. (1985) *Cell* **40,** 635–643

Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1990) *Current Protocols in Molecular Biology,* Wiley-Interscience, New York, pp. 9.2.1–9.2.6

Barbier-Brygoo, H., Ephritikhine, G., Klämbt, D., Ghislan, M., and Guern, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 891–895

Barbier-Brygoo, H., Ephritikhine, G., Klämbt, D., Maurel, C., Palme, K., Schell, J., and Guern, J. (1991) *Plant J.* **1,** 83–93

Battey, F. D., Gafvels, M. E., FitzGerald, D. J., Argraves, W. S., Chappell, D. A., Strauss, J. F., III, and Strickland, D. K. (1994) *J. Biol. Chem.* **269,** 23268–23273

Blatt, M. R., and Thiel, G. (1994) *Plant J.* **5,** 55–68

Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254

Brown, J. C., and Jones, A. M. (1994) *J. Biol. Chem.* **269,** 21136–21140

Chaudhary, V. K., Jinno, Y., FitzGerald, D., and Pastans, I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 308–312

Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108

Cleland, R. E. (1976) *Plant Physiol.* **58,** 210–213

Cross, J. W. (1991) *New Biol.* **3,** 813–819

Deikmann, W., Venis, M. A., and Robinson, D. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3425–3429

Edgerton, M. D., Tropsha, A., and Jones, A. M. (1994) *Phytochemistry* **35,** 1111–1123

Goldsmith, M. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11442–11445

Hertel, R., Thomson, K.-S., and Russo, V. E. A. (1972) *Planta* **107,** 325–340

Hesse, T., Feldswisch, J., Balshusemann, D., Bauw, G., Puype, M., Vanderkerckhove, J., Löbler, M., Klämbt, D., Schell, J., and Palme, K. (1989) *EMBO J.* **8,** 2453–2461

Hoffman, B., Planker, R., and Mengel, K. (1992) *Physiol. Plant.* **84,** 146–153

Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* **257,** 1496–1502

Inohara, N., Shimomura, S., Fukui, T., and Futai, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3564–3568

Jacobs, M., and Ray, P. M. (1976) *Plant Physiol.* **58,** 203–209

Jones, A. M. (1990) *Plant Physiol.* **93,** 1154–1161

Jones, A. M. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45,** 393–420

Jones, A. M., and Herman, E. (1993) *Plant Physiol.* **101,** 595–606

Jones, A. M., and Venis, M. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6153–6156

Jones, A. M., Melhado, L. L., Ho, T.-H. D., and Leonard, N. J. (1984) *Plant Physiol.* **74,** 294–302

Jones, A. M., Lamerson, P., and Venis, M. A. (1989) *Planta* **179,** 409–414

Jones, A. M., Cochran, D. S., Lamerson, P. M., Evans, M. L., and Cohen, J. D. (1991) *Plant Physiol.* **97,** 352–358

Klämbt, D. (1990) *Plant Mol. Biol.* **14,** 1045–1050

Kornfeld, S. (1992) *Annu. Rev. Biochem.* **61,** 307–330

Kounnas, M. Z., Argraves, W. S., and Strickland, D. K. (1992a) *J. Biol. Chem.* **267,** 21162–21166

Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., and Saelinger, C. B. (1992b) *J. Biol. Chem.* **267,** 12420–12423

Löbler, M., and Klämbt, D. (1985) *J. Biol. Chem.* **260,** 9848–9853

Macdonald, H., Henderson, J., Napier, R. M., Venis, M. A., Hawes, and Lazarus, C. M. (1994) *Plant Physiol.* **105,** 1049–1057

Melhado, L. L., Pearce, C. J., D'Alarco, M., and Leonard, N. J. (1982) *Phytochemistry* **21,** 2879–2885

Napier, R. M., and Venis, M. A. (1990) *Planta* **182,** 313–318

Napier, R. M., Venis, M. A., Bolton, M. A., Richardson, L. I., and Butcher, G. W. (1988) *Planta* **176,** 519–526

Napier, R. M., Fowke, L. C., Hawes, C., Lewis, M., and Pelham, H. R. B. (1992) *J. Cell Sci.* **102,** 261–271

Orlando, R. A., Kerjaschki, D., Kurihara, H., Biemesderfer, D., and Farquhar, M. G. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6698–6702

Picard, D., and Yamamoto, K. R. (1987) *EMBO J.* **6,** 3333–3340

Pietromonaco, S., Kerjaschki, D., Binder, S., Ullrich, R., and Farquhar, M. G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1811–1815

Pilarsky, C., and Koch-Brandt, C. (1992) *Eur. J. Cell Biol.* **59,** 275–279

Ray, P. M. (1977) *Plant Physiol.* **59,** 594–599

Ray, P. M., Dohrmann, U., and Hertel, R. (1977) *Plant Physiol.* **60,** 585–591

Ritter, J. K., Yeatman, M. T., Kaiser, C., Gridelli, B., and Owens, I. S. (1993) *J. Biol. Chem.* **268,** 23573–23579

Ronne, H., Ockland, C., Wiman, K., Rask, L., Obrink, B., and Petterson, P. A. (1983) *J. Cell Biol.* **96,** 907–910

Rothman, J. E., and Orci, L. (1992) *Nature* **355,** 409–415

Rück, A., Palme, K., Venis, M. A., Napier, R. M., and Felle, H. H. (1993) *Plant J.* **4,** 41–46

Shimomura, S., Sotobayashi, T., Futai, M., and Fukui, T. (1986) *J. Biochem. (Tokyo)* **99,** 1513–1524

Shimomura, S., Inohara, N., Fukui, T., and Futai, M. (1988) *Planta* **175,** 558–566

Shreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 2247–2258

Strickland, D. K., Ashcom, J. D., Williams, S., Battey, F., Behre, E., McTigue, K., Battey, J. F., and Argraves, W. S. (1991) *J. Biol. Chem.* **266,** 13364–13369

Strickland, D. K., Kounnas, M. Z., Williams, S. E., and Argraves, W. S. (1994) *Fibrinolysis* **8,** 204–215

Thiel, G., Blatt, M. R., Fricker, M. D., White, I. R., and Millner, P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11493–11497

Tillmann, U., Viola, G., Kayser, B., Siemiester, G., Hesse, T., Palme, K., Löbler, M., and Klämbt, D. (1989) *EMBO J.* **8,** 2463–2467

Wang, X., and Moore, T. S., Jr. (1991) *J. Biol. Chem.* **266,** 19981–19987

Wilson, D. W., Lewis, M. J., and Pelham, H. R. B. (1993) *J. Biol. Chem.* **268,** 7465–7468

Yilla, M., Tan, A., Ito, K., Miwa, K., and Ploegh, H. L. (1993) *J. Biol. Chem.* **268,** 19092–19100

Yoo, S. H., and Lewis, M. S. (1992) *J. Biol. Chem.* **267,** 11236–11241

Zimmermann, S., Thomine, S., Guern, J., and Barbier-Brygoo, H. (1994) *Plant J.* **6,** 707–716