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Abscisic Acid Binding to Extracts from Normal and Viviparous-1 Mutant Aleurone Layers of *Zea mays* L.

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Aleurone layers of normal and vp1 mutant maize kernels were extracted and centrifuged at 100,000 *g* to yield a cytosol fraction. Binding of [^{3}H]ABA *cis, trans* (+)ABA to a soluble macromolecular components present in the cytosol was demonstrated by Sephadex chromatography and non-denaturing PAGE. The binding component was of high molecular weight and seems to be an aggregate of proteins. A rapid DEAE-cellulose filter method for assaying bound [^{3}H]ABA to a soluble protein was adapted. Binding assays were performed with cytosol that had been preheated or incubated with several enzymes, indicating that heat and protease treatments disrupted the binding. This suggested that binding occurred to proteins. Some properties of the ABA binding proteins were described. The [^{3}H]ABA binding were reduced dramatically when unlabeled ABA was added as a competitor, suggesting a specific binding of [^{3}H]ABA. Gel filtration profiles and autoradiogram of [^{3}H]ABA binding showed no difference in the binding components of Vp1 and vp1/vp1 mutant cytosol, indicating that Vp1 protein is not a sole ABA binding protein.

Key words : maize, viviparous, ABA, aleurone, binding protein

The considerable progress made in purifying a number of plant hormone binding proteins and in producing cDNA clones promotes our understanding of the mechanisms underlying plant hormone action. Developments in the area of plant hormone receptors during last few years have been reviewed (Napier and Venis, 1990). Despite its remarkable roles during seed development, germination and during the response of plant to water stress, etc., the nature of abscisic acid(ABA) binding proteins has not been investigated except in the *Vicia faba* guard cell (Hornberg and Weiler, 1984). During maize seed development, the seed specific Vp1 gene product is required for ABA signal transduction related to the anthocyanin pigmentation of aleurone layers (McCarty *et al.*, 1989), and seed dormancy (Neil *et al.*, 1986; Robichaud and Sussex, 1987). Among the viviparous mutants(vp) of maize, vp1 mutant is unique in that it does not affect ABA synthesis in the seed (Fong *et al.*, 1983) or ABA metabolism (Robichaud and Sussex, 1987). However, vp1 embryo exhibits reduced sensitivity to ABA (Robichaud *et al.*, 1980). On this basis including the pleiotropic effects of Vp1 gene (Dooner, 1985) and its involvement in ABA regulated gene expression, Vp1 protein has been suggested as an ABA receptor (Robichaud *et al.*, 1980), or as an indirect effector which may potentiate the ABA response by interacting with one or more ABA-regulated transcription factors (McCarty *et al.*, 1991). As the partial separation in ABA and Vp1 regulatory function in maize embryo protoplast was reported (Hattori *et al.*, 1992), the possibility of Vp1 protein to be an ABA receptor was disproven indirectly.

Many plant genes were recently reported to respond to phytohormone ABA during seed develop-

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ment, and their ABA responding elements(ABRE) were also identified (reviewed by Skriver, and Mundy, 1990). The leucine zipper protein (EMBP-1) from mature wheat embryos was identified as a binding protein to the ABRE of the wheat Em gene (Guiltinan *et al.*, 1990). But the direct relationship between this protein and ABA was not elucidated yet.

In the present study, we have used a rapid, sc nsitive and reliable DEAE-cellulose filter assay, which was used originally for the identification of GA binding protein, to investigate the ABA binding proteins of maize aleurone cytosol. Using normal (Vp1) and mutant (vp1/vp1) aleurone layers, we present evidence that Vp1 protein is not a single, unique component for ABA binding protein. Also we describe the characteristics of [^{3}H](+)ABA binding proteins in preliminary form.

MATERIALS AND METHODS

Plant material

The lines of maize (*Zea mays* L.) used in this study were in the genetic background of the inbred TX-5855. The viviparous-1(Vp1) materials carry all the complementary factors required for anthocyanin pigmentation in the aleurone, (Vp, A_1, A_2, C, R and P_r). Homozygous recessive vp1 mutant kernels can be segregated from ears of self-pollinated heterozygotes on the basis of aleurone color by 15 days after pollination (DAP) prior to plumule expansion. These genetic mutant lines were grown on the Texas Agricultural Experiment Station's farm (Texas A & M Univ.) under routine conditions. At 20 DAP, all ears segregating the wild and mutant kernels (Vp1 blue, vp1/vp1 colorless) were harvested at mid-day and taken to the laboratory where they were processed immediately or frozen in liquid N_2 and stored at −70℃ for later use.

Radiochemical and chemicals

[^{3}H] (+)*cis*, *trans* ABA(18.2 Ci/ mmole) was obtained from Amersham. Sepadex G-200 was product of Pharmacia Fine Chemicals. DEAE-cellulose filters (DE81) were obtained from Whatman, and unlabelled (+)*cis*, *trans* ABA and other chemicals from Sigma.

Protein extraction

Total protein extracts from aleurone tissue were prepared according to Keith *et al.* (1982) with minor modifications. Colored or colorless aleurone layers were dissected from the endosperm on ice and all the following procedures were carried at 0-4℃. Aleurone tissues were ground with pestle and mortar in an equal volume (1 : 1 w/v) of extraction buffer [10 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 2.5 M phenylmethanesulfonyl fluoride (PMSF), pH 7.5]. The extract was filtered through eight layers of cheesecloth and centrifuged 1 h at 100,000 *g* using Beckman SW40.1 rotor. The supernatant was either used directly in the binding assay, or distributed to several tubes, frozen in liquid N_2 and stored at −70℃. All protein measurements were made by using Pierce protein assay reagent.

Filter assay

In the initial screening for ABA-binding protein, 30-40 μL of cytosol (containing 300-400 μg of soluble protein in extraction buffer) was mixed with [^{3}H] (+)ABA to a final concentration of 3-10 μM, which was identified as an optimal ABA concentration to induce the anthocyanin pigmentation in maize kernel culture (Hole *et al.*, 1989). Methanol in [^{3}H] ABA was evaporated with speed vacuum and [^{3}H] ABA was resuspended in same volume of buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). After incubation for 5-8 h, a 50 μL aliquot was assayed for the perspective bound hormone by DEAE-cellulose filter (Keith *et al.*, 1982). Parallel incubations were performed in which a 100-fold excess of unlabelled (+)ABA was added simultaneously with the labelled ABA as a competitor to determine the specific binding component. Also the binding of [^{3}H](+)ABA to the same amount of BSA (fraction *V*) as the protein amount of the cytosol was performed as a control. All the handling of ABA was carried out in the dark. Samples were assayed in triplicate. The filtration assay for the bound [^{3}H](+)ABA was performed as follow. Two DEAE-cellulose filter discs (diameter 2.5 cm) were soaked in assay buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and inserted

into a Millipore filter manifold. The filters were rinsed with 20 mL of assay buffer, the vacuum was released, and a 50 μL aliquot of sample was applied. After 1 min, 50 mL of assay buffer was drawn through the filters by suction to remove unbound [^{3}H]ABA. The discs were allowed to dry after the wash, then were placed in scintillation vials containing 1 mL methanol. After 30 min extraction, radioactivity was measured in Beckman liquid scintillation counter.

Gel filtration

All chromatographic procedures were performed at 4℃ in dark in which 0.5 mL of Vp1 or vp1/vp1 cytosol (5 mg of protein) was mixed with [^{3}H](+)ABA to a final concentration of 10 μM. After incubation for 12 h at 23℃ under the dark condition, the extract was loaded onto a 45×1.6 cm bed of Sephadex G-200. Columns were eluted under gravity with elution buffer (10 mM Tris-HCl, 1 mM EDTA, 2.5 M PMSF, pH 7.5) at a flow rate of 10 mL h^{-1}. Two mL fractions were collected. One mL aliquot of the fractions were mixed with 9 mL of BCS (Amersham) to count radioactivity. 100 μL aliquot of each fraction was used for protein measurement. Blue dextran (mol. wt. 2,000,000) and [^{3}H](+)ABA were used as a marker.

Non-denaturing PAGE

The fractions showing the highest radioactivity in gel filtration experiments were chosen as samples for electrophoresis. The fraction number of 10 for Vp1 or 11 for vp1/vp1 showed the highest radioactivity.

The samples corresponding to 2×10^3 cpm radioactivity (5.5 μg of protein for Vp1 or 12 μg of protein for vp1/vp1 fraction) were separated in a 4% polyacrylamide gel (30% acrylamide, 0.8% bisacrylamide) containing 0.375 M Tris-HCl (pH 8.8). The gel was run at 10 mV for overnight under the cold (4℃) and dark conditions. At the end of electrophoresis, the gel was treated with EN^{3}HACE autoradiography enhancer (New England Nuclear) as manufacturer's manual. The gel was dried using a gel dryer (Bio-Rad) and autoradiographed using Kodak X-Omat AR film with enhancing screen for 7 days.

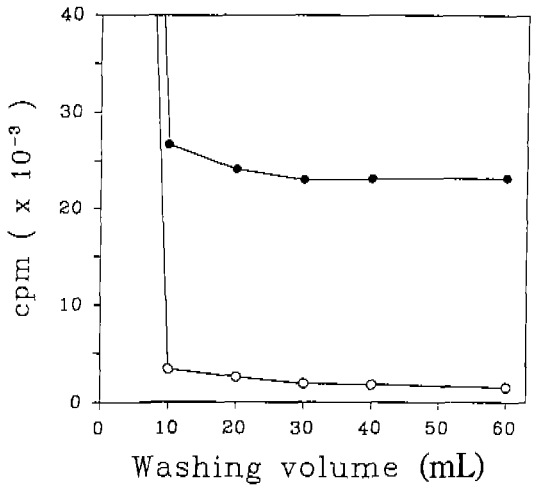

Fig. 1. Removal of unbound [^{3}H](+)ABA from DEAE-cellulose filters as a function of wash volume. Twenty of 30 μM [^{3}H](+)ABA in extraction buffer (○) (1.74×10^5 cpm) were applied to stacks of two filters and washed with increasing volumes of 10 mM Tris-HCl, 1 mM EDTA buffer (pH 7.5). Seventy five of 100,000 *g* Vp1 supernatant (75 μg) was mixed with 20 μL of [^{3}H] ABA (●) and incubated in dark conditions for 5 h at 4℃, then applied to filters.

RESULTS

A 100,000 *g* cytosol was prepared from wild (Vp1) and mutant (vp1/vp1) aleurone of maize. Binding of [^{3}H](+)ABA to each 100,000 *g* cytosol was investigated by filter assay, gel filtration and non-denaturing PAGE.

Sensitivity of filter disc assay

Initial experiments were performed to determine whether the filter assay used for gibberellin binding (Keith *et al.*, 1982) is a reliable method for assaying bound ABA. Fig. 1 showed that when [^{3}H](+)ABA and BSA mixture was applied to two DEAE cellulose filters as control, washing with 60 mL of assay buffer was sufficient to remove most of the unbound radioactivity. Less than 1% of the applied radioactive materials left on the filters. However, [^{3}H]ABA bound to 100,000 *g* soluble proteins remained on the filters even after washing with 60 mL of buffer. Most of the unbound radioactivity was removed by washing with 20 mL buffer. When the same amount

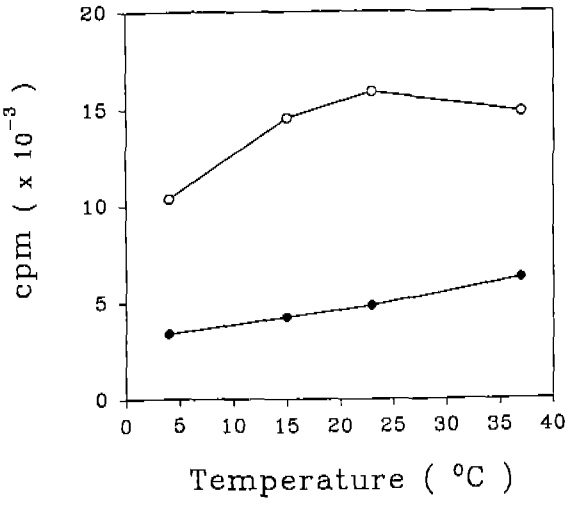

Fig. 2. The effect of incubation temperatures on levels of [^{3}H](+)ABA binding to 100,000*g* Vpl cytosol (○) or BSA (●). A hundred fifty of soluble protein was incubated in 4.5 μM [^{3}H](+)ABA for 5 h.

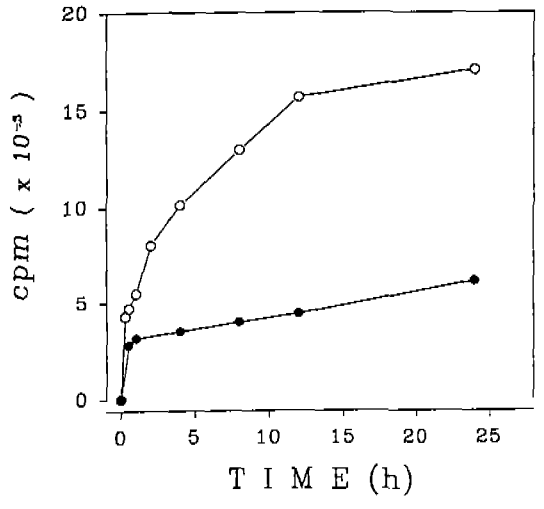

Fig. 3. Time-course of [^{3}H](+)ABA binding to 100,000*g* cytosol of Vpl aleurone (○) or BSA (●). A hundred fifty of soluble protein was incubated in 4.5 μM [^{3}H](+)ABA.

of BSA was used, the radioactivity remained on the filters was same as control [^{3}H]ABA.

Optimal conditions for binding

The effect of experimental conditions on [^{3}H](+)ABA binding was tested by determining the level of binding to 100,000*g* Vpl cytosol. One hundred and fifty μg of cytosol proteins or BSA was mixed with [^{3}H](+)ABA to a final concentration of 4.5 μM and incubated in the dark at different temperatures. After 5 h incubation, 50 μL aliquot of sample was filter assayed (Fig. 2). While binding of [^{3}H](+)ABA to Vpl cytosol increased with increasing temperature, showing greatest binding at 23℃ and decreased at 37℃, binding to BSA continued to increase up to 37℃. In experimints described so far, incubation of cytosol with [^{3}H](+)ABA was performed for 5 h. An experiment was carried out in which level of total binding were determined after various time periods up to 24 h. Fig. 3 indicates that binding of [^{3}H](+)ABA increased with longer incubation time, but the slope of increment lowered after 12 h in Vpl cytosol. In case of BSA, [^{3}H](+)ABA binding did not change much in incubation period.

Table 1. Competition of unlabelled (+)ABA with [^{3}H](+)ABA in binding to 100,000*g* cytosol or BSA

	Vpl Cytosol[a]		BSA[a]	
	cpm	%	cpm	%
[^{3}H](+)ABA control	16,260	100	6,160	100
+10 fold (+)ABA	9,200	57	4,870	94
+60 fold (+)ABA	6,560	40	4,570	89
+120 fold (+)ABA	5,700	35	4,330	84

[a]One hundred and fifty μg of soluble protein was incubated with [^{3}H](+)ABA to a final concentration of 4 μM with or without unlabelled (+)ABA of different concentrations at 23℃ for 8 h.

Specificity of binding

The specificity of binding was tested by incubating 150 μg of Vpl cytosol protein or BSA in 4 μM [^{3}H](+)ABA with different concentration of unlabelled (+)ABA as a competitor. As Table 1 shows, 120 fold concentration of unlabelled (+)ABA significantly reduced the [^{3}H](+)ABA binding in Vpl cytosol to 35% of control, but in BSA, unlabelled (+)ABA did not inhibit the binding of [^{3}H](+)ABA effectively even in 120 fold concentration. The 100,000*g* cytosol of mutant (vp1/vp1) was also tested for specific binding to [^{3}H](+)ABA and showed the very same levels of radioactivity and reduction rate as Vpl cytosol did (data not shown). Competition of unlabelled (+)ABA with [^{3}H](+)ABA in binding to BSA protein was used as a control for nonspecific binding.

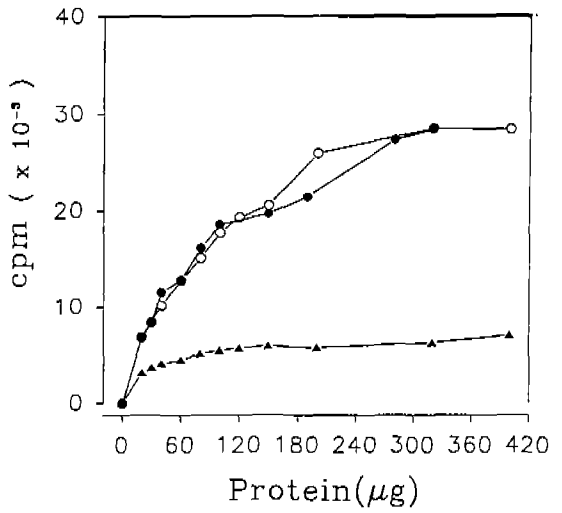

Fig. 4. Linear relationship between specific [^{3}H](+) ABA binding and protein concentration. 100,000 *g* cytosol preparation of Vp1 (○), mutant (vp1/vp1) aleurone (●) and BSA protein (▲) were incubated 8 h at 23℃ with 6 μM [^{3}H](+)ABA in the presence (A) and absence (B) of 10 fold excess unlabelled (+)ABA. Specific bin ding was calculated by substracting the nonspecific bi nding value (A) from total binding (B).

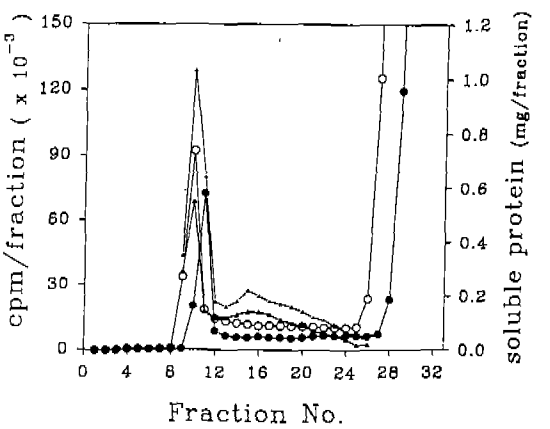

Fig. 5. Gel filtration profile of [^{3}H](+)ABA binding components in 100,000 *g* cytosol of Vp1 (●) or vp1/vp1 (○). Cytosol samples were prepared from aleurone tis sues of 20 DAP Vp1 or vp1/vp1 maize kernels. Five hundred of cytosol samples referring to 5 mg of protein was incubated with [^{3}H](+)ABA (10 μM) for 12 h at 23℃. Separation was performed under the dark condition in the cold room (4℃). One hundred μL aliquot of each fraction was used for protein measurement, Vp1 (▲), vp1/vp1 (△).

Table 2. Effect of heat and enzymes on [^{3}H](+)ABA binding to 100,000 *g* cytosol

Treatment[a]	[^{3}H](+)ABA specific binding (cpm mg^{-1} protein)	% of control
Control	71,110	100
DNase 1/RNase A	51,910	73
Proteinase K	7,140	10
Trypsin	4,830	6.8
Heat (65℃ for 1 h)	51,400	72
Heat (>90℃ for 15 min)	4,200	6
Phospholipase A_2	70,400	99[b]

[a]Fifty μL aliquot of Vp1 cytosol was treated with no addition (control) or with addition of enzymes at 23℃ for 1 h, and mixed with [^{3}H](+)ABA to a final concentration of 4 μM. Incubation was done in the dark for 8 h at 23℃. [b]Phospholipase A_2 showed an increase in binding occasionally.

Heat and enzyme treatments

Several different treatments to the cytosol were performed to test wheather the cytosol component bound specifically to ABA was protein. Aliquots (5 0 μL) of Vp1 cytosol were incubated for 1 h at 23℃ with (a) no addition, (b) 50 μg/mL DNase/RNase A plus 10 μM $MgCl_2$, (c) 1 mg mL^{-1} proteinase K (d) 1 mg mL^{-1} trypsin, (e) 1 mg mL^{-1} phospholipase A_2. An additional sample was heated for 1 h at 65℃ or heated for 15 min at higher than 90℃ and then cooled in ice. [^{3}H](+)ABA was added to each sample in the concentration of 4 M. All samples were incubated 8 h at 23℃ in the dark. Trypsin and proteinase K were completely effective in abolishing binding, and heat treatment to 90℃ also significantly reduced the binding activity of cytosol component (Table 2), suggesting that binding was occurring to a protein. However, 65℃ heat treatment had only a small effect in reducing binding level.

Linearity of specific [^{3}H](+)ABA binding with protein concentration

The linearity of specific ABA binding with soluble protein concentration was tested. Fig. 4 showed that specifically bound (+)ABA measured in filter assay in linear between 20 and 100 μg of soluble protein. Binding of [^{3}H](+)ABA to both Vp1 and vp1/vp1 cytosol was linear over this range and showed the same slope of linearity. However the spcific binding of ABA to BSA did not change as

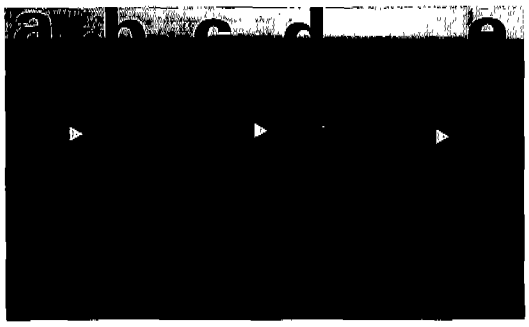

Fig. 6. Autoradiogram showing [^{3}H](+)ABA binding to 100,000 *g* cytosols from normal (Vp1) [a, b] or mutant (vp1/vp1) [d, e] aleurone of 20 DAP maize kernel. Proteins and free [^{3}H](+)ABA [c] were separated by 4% non-denaturing PAGE.

protein concentration increased.

Separation of bound radioactivity

Binding of [^{3}H]ABA to 100,000 *g* cytosol from Vp1 or vp1/vp1 aleurone layers was investigated by Sephadex G-200 gel filtration (Fig. 5). [^{3}H](+)ABA binding profile of Vp1 cytosol was very similar to that of vp1/vp1 cytosol. Both of the normal and mutant cytosol showed the bulk of the total binding occurred right after the void volume of the gel bed, indicating the size of the binding components to be huge.

Identification of the [^{3}H](+)ABA binding components

In order to identify the binding components non-denaturing PAGE was performed using the samples of gel filtration experiments. Fig. 6 showed the autoradiogram of the [^{3}H](+)ABA binding components in Vp1 and vp1/vp1 cytosol. The binding components of normal and mutant cytosol appeared in the same region of the non-denaturing gel, and seemed to have large molecular size as expected from the gel filtration results.

DISCUSSION

The results reported here indicate that [^{3}H](+) ABA binds *in vitro* to a soluble protein present in both of the normal (Vp1) or colorless mutant (vp 1/vp1) aleurone layers of maize kernels. The termi nology for binding used in this paper was adapted from that advocated previously (Ray *et al.*, 1977. Sussman and Kende, 1978). Specific binding of [^{3}H](+)ABA which is saturable, reversible and of high affinity, is distinguishable from the nonspecific, low affinity binding that persists in the presence of excess nonradioactive (+)ABA. The DEAE filter assay, which was developed for *in vitro* binding of GAs, seems to be suitable for ABA binding assay judging from the results of several test, preliminary was sufficiently removed through washing with 60 mL of assay buffer, bound [^{3}H](+)ABA to the 100,000 *g* cytosol remained on the filters after washing (Fig. 1). ABA binding to the cytosol changed at different temperatures showing maximum binding at 23℃, but the binding to BSA continued to increase up to 37℃ (Fig. 2). Competitive binding of unlabelled ABA to BSA didn't lower the bound level of [^{3}H](+)ABA, but did reduce the bound level of [^{3}H](+)ABA to 35% in cytosol (Table 1). Taken together, it might be explained such that total binding of cytosol components reduced due to the loss of specific binding at 37℃, but nonspecific binding to BSA increased (Keith *et al.*, 1981). Comparing to the reports that specific binding of GAs to maize coleoptile or cucumber hypocotyl cytosol reached on equilibrium within 2 h (Keith *et al.*, 1981, Keith and Rappaport, 1987), we observed ABA binding to maize aleurone cytosol reached an equilibrium after 12 h. To evaluate the result, more data on the half-time association and exchangibility with unlabelled ABA might be needed. Specifically bound ABA was linear betweeen 20 and 100 μg soluble protein of normal and mutant cytosol, which was same range of linearity for GA binding in filter assay (Keith *et al.*, 1982). As shown in Table 2, the specifically bound portion of ABA binding to cytosol components was significantly reduced by heat or protease treatment, suggesting that binding was occurring to proteins.

Hattori *et al.* (1992) showed, using the GUS re porter plasmid electroporated to maize protoplast that the promoter region of C1 gene had two consensus sequences, ABRE responding to ABA and Vp1 protein and Sph element mainly responding to Vp1 protein. However, it was not explained how ABA and Vp1 protein activated the C1 gene promoter; through second messenger pathway or dire-

ctly by ABA and its binding protein with other transcriptional factor. On the other hand, ABA was known to regulate the catalase-2 gene expression without the Vp1 protein during maize seed germination (Williamson and Scandalios, 1992). Therefore, we assumed that Vp1 protein was not involved in the ABA binding complex formation anyhow. Around 17 DAP, normal maize kernels, Vp1, start to accumulate the anthocyanin pigment in aleurone tissues under the control of ABA (Smith *et al.*, 1991). From the relationship between ABA and Vp 1, therefore, it would be reasonable to investigate the possibility of Vp1 protein to be a binding component of ABA using ABA-insensitive vp1 mut ant at this developmental stage, 20 DAP. Gel filtration profiles of ABA binding components in normal and mutant cytosol appeared very similar each other (Fig. 6). Three proteins from the guard cell protoplast of *Vicia faba* were reported to be cross-linked specifically with *cis*(+)ABA, but separation of these in non-denaturing conditions was not analyzed (Hornberg and Weiler, 1984). Taken the results together, the ABA binding components in maize aleurone cytosol is a high molecular weight protein which seems to be an aggregate of at least more than three proteins. Our preliminary test on the binding proteins on SDS-PAGE showed three proteins pattern (data not shown). There are no differences between the ABA binding proteins of normal and vp1 mutant cytosol, in the Sephadex ch romatography and gel separations, suggesting that Vp1 protein is at least not a unique ABA receptor protein. In other words, the ABA binding protein might not be related to Vp1 protein. However, we cannot rule out the possibility of Vp1 protein involvement through protein-protein interactions in the ABA regulating events during seed development at this time. In order to distinguish whether the 90 kD Vp1 protein (McCarty *et al.*, 1991) is included in the ABA binding protein aggregate or not, these aggregates from normal and mutant must be analyzed. The nature of the ABA binding protein of maize aleurone tissues and its biological functions must be studied soon. We clearly showed that the filter assay for GA binding protein can be successfully extended for ABA binding protein, even though its kinetics, interactions with hormone analogs and some of its properites are not fully investigated.

ACKNOWLEDGMENT

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옥수수 胎生突然變異體-1 및 正常體의 糊粉層 抽出物과 ABA의 結合

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적 요

정상체 옥수수 종자와 태생 돌연변이체(vp1) 종자의 호분층으로부터 단백질을 추출하고, 100,000 *g*에서 원심분리 후 상징액을 사용하여 [^{3}H](+)ABA와의 결합 여부를 조사하였다. Sephadex 크로마토그라피와 비변성 PAGE에 의하여 각 호분층의 상징액에 [^{3}H] *cis, trans*(+)ABA와 결합하는 거대 물질의 존재를 확인하였다. ABA와 결합한 성분의 특성을 조사하기 위하여 지베렐린 결합 단백질 조사에 사용되었던 DEAE-cellulose filter 분석법을 적용시켰다. ABA 결합 물질의 특성을 조사하기 위하여 호분층 상징액을 열과 여러 효소로 처리하여 본 결과, 열(90℃ 이상)과 단백질 분해 효소로 처리하였을 경우, ABA와의 결합이 급격히 감소된 것으로 미루어 ABA 결합 물질이 단백질임을 판명하였다. 방사선 동위 원소가 입혀지지 않은 ABA를 경쟁 물질로 사용하였을 때, [^{3}H]ABA의 결합이 또한 급격히 감소되는 점으로 미루어 [^{3}H]ABA의 결합은 무작위적인 결합이 아닌 특수 결합임을 확인하였다. 정상체와 돌연변이체의 호분층 상징액에 존재하는 ABA 결합 단백질들을 젤 여과법과 비변성 PAGE 방사선 사진의 결과로 비교하였을 경우 차이점을 발견할 수 없었다. 이 결과로부터 ABA 결합 단백질은 Vp1 단백질로만 구성되지는 않았을 것으로 사료된다.

주요어 : 옥수수, 태생돌연변이, ABA, 호분층, 결합단백질

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