The Plant Cell, Vol. 6, 1681–1692, November 1994 © 1994 American Society of Plant Physiologists

A Fusicoccin Binding Protein Belongs to the Family of 14-3-3 Brain Protein Homologs

Henrie A. A. J. Korthout and Albertus H. de Boer[1]

Department of Plant Physiology and Biochemistry, Institute of Molecular Biological Sciences, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

The fusicoccin binding protein (FCBP) is a highly conserved plasma membrane protein present in all higher plants tested thus far. It exhibits high- and low-affinity binding for the fungal toxin fusicoccin (FC). We purified the active FCBP from a fraction highly enriched in plasma membrane by selective precipitation and anion exchange chromatography. After SDS-PAGE, the two FCBP subunits of 30 and 31 kD were detected as major bands. Amino acid sequence analysis of the 31-kD polypeptide displayed a high degree of identity with so-called 14-3-3 proteins, a class of mammalian brain proteins initially described as regulators of neurotransmitter synthesis and protein kinase C inhibitors. Thereafter, we affinity purified the 30- and 31-kD FCBP subunits, using biotinylated FC in combination with a monomeric avidin column. Immunodecoration of these 30- and 31-kD FCBP subunits with polyclonal antibodies raised against a 14-3-3 homolog from yeast confirmed the identity of the FCBP as a 14-3-3 homolog. Similar to all 14-3-3 protein homologs, the FCBP seems to exist as a dimer in native form. Thus far, the FCBP is the only 14-3-3 homolog with a receptor-like function. The conserved structure of the 14-3-3 protein family is a further indication that the FCBP plays an important role in the physiology of higher plants.

INTRODUCTION

Fusicoccin (FC), a metabolite of the pathogen *Fusicoccum amygdali*, was isolated nearly three decades ago as the causative agent in the wilting of peach and almond trees infected by the pathogen (Ballio et al., 1964). The wilting of leaves is caused by an irreversible opening of the stomata as a result of osmotic swelling of the guard cells. Increased water uptake by the guard cells is the consequence of FC-induced activation of the H^+-pump in the plasma membrane and increased K^+ and Cl^- uptake (Marrè, 1979). FC has other physiological effects as well, thereby mimicking or antagonizing natural plant hormones, such as auxin, abscisic acid, and gibberellic acid (Marrè, 1979), and light (Assman and Schwartz, 1992).

Since the first report on the presence of an FC binding protein (FCBP) with high binding affinity for FC (Dohrman et al., 1977), research has focused on the properties of this receptor (Weiler et al., 1990). The FCBP was purified to homogeneity by means of FC affinity chromatography (de Boer et al., 1989; Korthout et al., 1994) as two polypeptides with apparent molecular masses of 30 and 31 kD. Aducci et al. (1988) demonstrated the receptor function of the FCBP by means of functional reconstitution of a crude FCBP preparation with one of its effector proteins, the plasma membrane H^+-ATPase. The FCBP is exclusively found in the plasma membrane. Binding activity in the plasma membrane is cell-type specific (Meyer et al., 1993) and is controlled by the developmental stage of the tissue (Basel and Cleland, 1992). Modulation of the receptor affinity for FC by binding of its own ligand (Basel et al., 1994) further accentuates the role of the FCBP in the physiology of plants.

The very wide distribution of the FCBP in plants (Meyer et al., 1993) is a seeming contradiction with the restricted distribution of the FC-producing pathogen *F. amygdali.* Therefore, several attempts have been made to find an endogenous ligand eliciting a similar or opposite response to that of FC. A number of independent groups have reported the existence of a FC-like factor in plants (Aducci et al., 1980; Weiler et al., 1990). Muromtsev et al. (1989, 1994) reported the presence of FC A and C in the kernel of maize. The low abundance of the FC-like factor(s) in plant tissue, which is in line with the very high affinity of the FCBP for FC, has thus far hampered the elucidation of its structure. However, this seems just a matter of time, as with gibberellin. Gibberellin was initially isolated from a culture of the pathogenic fungus *Gibberella fujikuroi*, and subsequently it took more than 25 years to determine that it was an important plant hormone.

The physiological relevance of the FC signal transduction pathway will be understood from the nature of the effector proteins, which are all integral plasma membrane proteins. The primary target is the H^+-ATPase providing the electrochemical gradient for secondary transport processes across the plasma membrane (Marrè, 1979). Another P-type pump in the plasma membrane, the Ca^{2+}-ATPase that plays a role

[1] To whom correspondence should be addressed.

in Ca^{2+} homeostasis, is also modulated by FC (Liss et al., 1991). Outward rectifying K^+ channels are clearly inhibited by FC (Blatt and Clint, 1989). For the H^+-ATPase, a mechanism for FC modulation is emerging (Johansson et al., 1993; Rasi-Caldogno et al., 1993; Lanfermeyer and Prins, 1994): FC releases the restriction imposed on the H^+-pump by the autoinhibitory C-terminal domain. How the restriction is released is not yet known, but it might involve kinase/phosphatase-mediated phosphorylation. The C terminus of the plant H^+-ATPase has several potential phosphorylation sites, and from oat roots, a Ca^{2+}- and phospholipid-dependent kinase that phosphorylates the H^+-ATPase has been partially purified (Schaller et al., 1992). Recently, Vera-Estrella et al. (1994) reported that dephosphorylation of the H^+-ATPase results in increased pump activity.

Investigations into the action of FC and its receptor have raised fundamental questions regarding (1) the function of the FCBP in higher plants, (2) how the modulation of a diverse class of transport proteins is brought about, (3) how FC increases the number of binding sites and the affinity of its own receptor, and (4) what role the receptor protein plays in other hormonal and light signal transduction pathways. The key to the FC "mystery" lies in the nature of the FCBP. In this study, we show that the FCBP belongs to the family of so-called brain regulatory proteins, also known as 14-3-3 proteins. The FCBP is unique among known 14-3-3 proteins in that it is a plasma membrane protein with characteristics of a receptor for extracellular signals. The potential of 14-3-3 proteins to regulate protein kinase C (Aitken et al., 1992) lends further support to our hypothesis that FC interferes with the action of a C-type protein kinase in oat (de Boer et al., 1994).

RESULTS

Purification of the 31-kD Subunit of the FCBP

To purify the FCBP in active form and in sufficient amount for sequence analysis, the purification protocol as depicted in Figure 1 was developed. The protocol comprises five important steps. In the first step, soluble cytoplasmic proteins were separated from the particulate fraction, the microsomes. In the cytoplasmic fraction, no ^{3}H-dihydrofusicoccin (^{3}H-FC) binding activity was found. In the second step, we purified plasma membrane vesicles from the microsomal fraction using the aqueous two-phase partitioning method because the FCBP is an integral plasma membrane protein. In this step, the vanadate-sensitive, K^+- and Mg^{2+}-dependent H^+-ATPase and the FCBP were enriched 11-fold as compared with the microsomal fraction. Before solubilization of the FCBP from the plasma membrane fraction, the binding sites were protected from degradation by binding of an FC conjugate (biotinylated FC). Biotinylated FC has a lower affinity for the binding site than ^{3}H-FC (dissociation constant of 70 versus 3 nM, respectively; Korthout et al., 1994), and ^{3}H-FC displaced ~25% of the bound biotinylated FC in 12 hr. Eighty percent of the plasma membrane ^{3}H-FC binding activity was solubilized in this step.

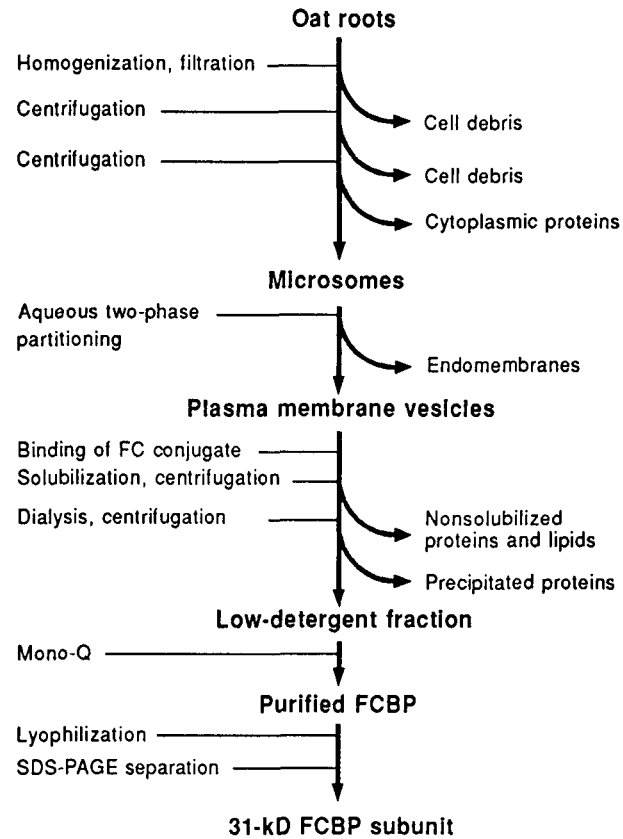

Figure 1. Flow Chart of the Purification of the FCBP.

The protocol describes the isolation of the 31-kD FCBP subunit from 6-day-old oat roots. Five steps are involved: differential centrifugation, aqueous two-phase partitioning, dialysis, anion exchange chromatography, and SDS-PAGE.

The third step in our purification protocol involved a selective precipitation of solubilized plasma membrane proteins at reduced detergent concentration and ionic strength. Approximately 75% of the detergent-solubilized proteins precipitated when we reduced the detergent concentration beyond the critical micelle concentration of octylglucoside (from 1.4 to 0.2%). Twenty-five percent of the proteins remained solubilized in the so-called "low-detergent" fraction. Approximately 65% of the plasma membrane ^{3}H-FC binding activity was recovered in the low-detergent fraction. Figure 2B shows the difference in polypeptide composition of the low-detergent (lane 1) and the precipitated (lane 2) fraction, after the dialysis step.

In the fourth step, the FCBP was highly enriched from the low-detergent dialysate by means of anion exchange chromatography (Mono-Q), as shown in Figure 2A. Two peaks containing the ^{3}H-FC binding activity eluted from the Mono-Q column at ~0.42 M KCl; no difference could be seen in the polypeptide pattern of both peak fractions after SDS-PAGE (data not shown). The purified FCBP appeared as a doublet with apparent molecular masses of 30 and 31 kD (Figure 2B, lane 3). These molecular masses of the FCBP subunits are

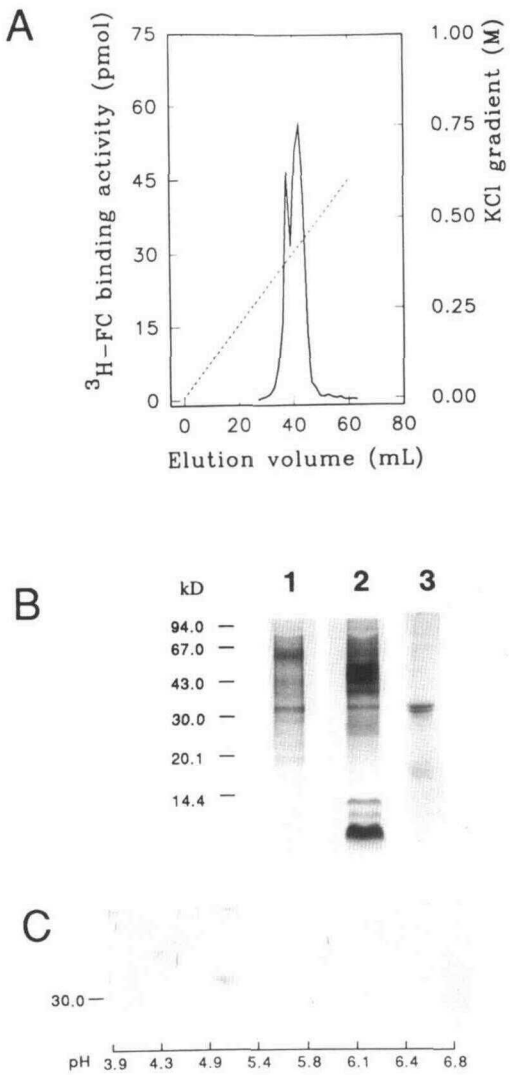

Figure 2. Isolation and Purification of the FCBP.

(A) Anion exchange chromatography of the low-detergent protein fraction by Mono-Q. Fractions, which were eluted within a 0 to 0.6 M KCl gradient (dashed line), were screened for ^{3}H-FC binding activity (solid line). Binding activity was eluted in two peaks at 0.40 and 0.44 M KCl, respectively.

(B) SDS-PAGE of samples obtained after the different purification steps. In lane 1, 15 μg of the low-detergent fraction was loaded; in lane 2, 10 μg of the precipitated protein fraction was loaded. Both fractions were obtained after selective precipitation by dialysis against low-detergent and low-salt buffer. Lanes 1 and 2 were stained with silver. Lane 3 shows the polypeptide pattern of the pooled Mono-Q fractions that showed ^{3}H-FC binding activity (fractions 34 to 44). This lane was stained with Coomassie blue. Numbers at the left indicate the positions of the molecular mass markers (in kilodaltons).

(C) Silver-stained two-dimensional gel of the 31-kD polypeptide as shown in lane 3 of **(B)**. The 31-kD band was excised from a polyacrylamide gel and electroeluted before loading on the two-dimensional gel. This polypeptide was used for sequence analysis. The position of the 30-kD molecular mass marker is given at the left.

identical to those of the doublet purified by FC affinity chromatography (de Boer et al., 1989; Korthout et al., 1994) and a combination of metal chelate and anion exchange chromatography (Oecking and Weiler, 1991). Fractions 36 to 46 (Figure 2A), representing 50% of the initial plasma membrane ^{3}H-FC binding activity, were pooled and lyophilized. Part of the lyophilized fraction, containing at least 25 pmol of FCBP, as calculated from ^{3}H-FC binding activity, was run on an SDS–polyacrylamide; from the gel, the 31-kD band was excised and used for sequence analysis.

To check the purity of the 31-kD band, proteins present in the excised 31-kD band were electroeluted from the gel and analyzed by two-dimensional gel electrophoresis. One spot with a pI of ~5 was observed, showing that the excised 31-kD band contained only one polypeptide (Figure 2C). A similar pI (4.9) of the FCBP was reported by Aducci et al. (1982). Loading more of the electroeluted protein on a two-dimensional gel caused smearing, which might be a result of the fact that the 31-kD subunit is glycosylated (*H.A.A.J. Korthout and A.H. de Boer, unpublished results*).

Sequence Analysis of the 31-kD FCBP

In view of the possibility that the N terminus of the FCBP is acetylated (as often happens with membrane proteins), we chose to obtain internal sequence information. The excised 31-kD polypeptide was digested in situ with trypsin, and the resulting peptides were fractionated by reverse phase HPLC. Figure 3 gives the primary amino acid sequence information obtained from three peptides. A search of the EMBL data base revealed that these sequences from the FCBP's 31-kD subunit are homologous to sequences from members of so-called brain regulatory proteins, also known as 14-3-3 proteins (Aitken et al., 1992). The alignment of the FCBP amino acid sequences with derived amino acid sequences of 14-3-3 proteins from sheep brain (Toker et al., 1992), yeast (van Heusden et al., 1992), *Oenothera hookeri* (Hirsch et al., 1992), *Arabidopsis* (Lu et al., 1992), and barley (Brandt et al., 1992) shows the high degree of similarity with all five polypeptides.

The 30-kD FCBP Subunit Is also a 14-3-3 Homolog

From three different species, using different methods, the FCBP has been purified as a doublet: 30 and 31 kD from oat root (de Boer et al., 1989; Korthout et al., 1994), 29.5 and 31.3 kD from broad bean leaves (Feyerabend and Weiler, 1989), and 30.5 and 31.6 kD from *Commelina communis* (Oecking and Weiler, 1991). Photoaffinity labeling experiments, using azido-labeled ^{3}H-FC, confirmed the FC binding capacity of both the 30- and 31-kD polypeptides (Oecking and Weiler, 1991). We addressed the question of whether the 30-kD subunit of the FCBP is also a member of the 14-3-3 protein family and, thus, closely related to the 31-kD subunit. Because all 14-3-3 proteins have extensive sequence homology (Aitken et al., 1992), we used polyclonal antibodies raised in rabbit against the

	37										
Sheep ε	Met	Asp	Val	...	...	Glu	Leu	Thr	Val	Glu	Glu	Arg
Yeast	Ser	Gly	Gln	...	...	Glu	Leu	Ser	Val	Glu	Glu	Arg
Oenothera	Ala	Ala	Asp	Ser	Glu	Glu	Leu	Thr	Val	Glu	Glu	Arg
Arabidopsis	Ala	Val	Asp	Gly	Asp	Glu	Leu	Thr	Val	Glu	Glu	Arg
Barley	Thr	Ala	Asp	Val	Gly	Glu	Leu	Thr	Val	Glu	Glu	Arg
Oat	Thr	Val	Asp	Ser	Glu	Glu	Leu	Thr	Val	Glu	Glu	*Arg*

	140						155						
Sheep ε	Tyr	Leu	Ala	Glu	Phe	Ala	Glu	Asn	Ser	Leu	Val	Ala	Tyr
Yeast	Tyr	Leu	Ala	Glu	Phe	Ser	Asn	Ala	Ser	Leu	Glu	Ala	Tyr
Oenothera	Tyr	Leu	Ala	Glu	Phe	Lys	Glu	Ser	Thr	Leu	Ser	Ala	Tyr
Arabidopsis	Tyr	Leu	Ala	Glu	Phe	Lys	Glu	His	Thr	Leu	Ala	Ala	Tyr
Barley	Tyr	Leu	Ala	Glu	Phe	Lys	Glu	Asn	Thr	Leu	Val	Ala	Tyr
Oat	Tyr	Leu	Ala	Glu	Phe	Lys	Glu	Asn	Thr	*Leu*	Val	Ala	Tyr

Figure 3. Amino Acid Homology of the Sequenced Peptides of the 31-kD FCBP Subunit with Members of the 14-3-3 Protein Family.

Alignment of the amino acid sequence of the FCBP 31-kD subunit with sequences of 14-3-3 protein homologs according to Aitken et al. (1992). Sequence 140 to 145 is part of a consensus sequence that is also found in annexins and that is likely the binding site for the regulatory domain of protein kinase C (Mochly-Rosen et al., 1991). The amino acids in italics are tentative assignments. Sequenator signals from two other peptides were rather low, but they could be brought into agreement with the 14-3-3 sequence from barley (Brandt et al., 1992): positions 68 to 72 (Ile-*Ile*-*Ser*-Ser-*Ile*) which are conserved in all 14-3-3 proteins and positions 206 to 211 (*Gln*-*Ala*-Phe-*Asp*-Gln-*Ala*). Dots indicate gaps introduced for sequence alignment.

BMH1 translation product of yeast, which is also a 14-3-3 protein (van Heusden et al., 1992). Figure 4 shows the resulting protein gel blot: the BMH1 antibodies clearly recognized the 31-kD subunit (thus confirming the outcome of the EMBL data base search) and the 30-kD subunit, both in a total plasma membrane fraction (Figure 4, lane 1) and in the purified FCBP fraction (lane 3).

Our conclusion is that the two polypeptides that make up the FCBP are closely related proteins. They could be isoforms, because other tissues are known to contain several isoforms of a 14-3-3 protein (de Vetten et al., 1992; Toker et al., 1992). On the other hand, the difference in molecular mass may reflect post-translational modifications like phosphorylation or glycosylation (H.A.A.J. Korthout and A.H. de Boer, unpublished results).

Cytoplasmic 14-3-3 Homologs Do Not Bind ^{3}H-FC

In total cell extracts from Arabidopsis and maize, 14-3-3 antibodies cross-react with at least five different polypeptides (de Vetten et al., 1992; Lu et al., 1992). Also, oat roots contain at least five polypeptides that cross-react with the BMH1 antibody: besides the two in the plasma membrane (Figure 4, lane 1), we found three polypeptides in the cytoplasmic protein fraction that cross-react with the BMH1 antibody (Figure 4, lane 2). The apparent molecular masses of the latter polypeptides are 27, 28, and 30.5 kD. These three 14-3-3 homologs are not involved in ^{3}H-FC binding because no binding activity was detected in the cytoplasm.

Immunodecoration with 14-3-3 Antibodies of the FC–Affinity Purified FCBP

To test unambiguously that the 14-3-3 homologs found in the plasma membrane are indeed subunits of the FCBP, we used a recently developed affinity purification method (Korthout et al., 1994). This method is based upon a bifunctional ligand: biotinylated FC. The advantage of the use of this ligand in combination with a monomeric avidin column is its specificity: with the FC moiety, the ligand binds specifically to the FCBP. The ligand plus FCBP then bind with the biotin moiety specifically to a monomeric avidin column, and finally, the FCBP/biotinylated FC complex is eluted selectively from the monomeric avidin column with excess biotin. Although the final yield is low, this method results in highly pure and active FCBP (Korthout et al., 1994).

Figure 5 shows that a peak of active FCBP was eluted from the avidin column with excess biotin; on a silver-stained SDS–polyacrylamide gel, the 30- and 31-kD bands were faintly visible in the fractions with the highest ^{3}H-FC binding activity (data

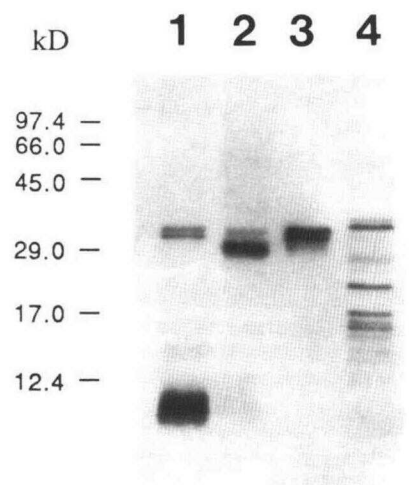

Figure 4. Immunological Relationship of the Two FCBP Subunits.

Protein gel blot analysis of total plasma membrane proteins from oat roots (3 μg, lane 1), total cytoplasmic proteins from oat roots (~2.5 μg, lane 2), the purified FCBP (25 ng, lane 3), and a total protein extract from yeast (5 μg, lane 4). The blot was immunodecorated with polyclonal antibodies raised against the *BMH1* gene translation product encoding a 14-3-3 homolog in yeast (BMH1 antibody). The 14-3-3 proteins were visualized on blot using the ECL method. Numbers at the left indicate the positions of the molecular mass markers (in kilodaltons).

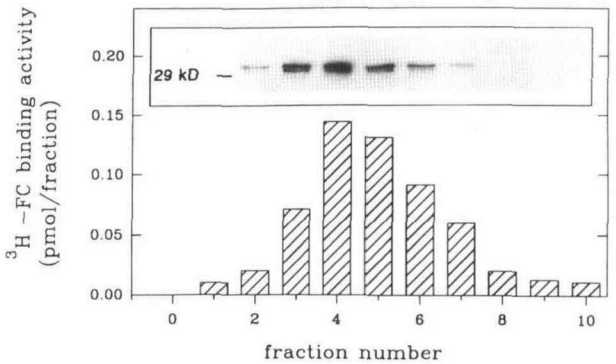

Figure 5. Immunodecoration of the Affinity-Purified FCBP Subunits with BMH1 Antibodies.

Plasma membrane vesicles (15 mg) of oat roots were charged with biotinylated FC, solubilized, and applied to a 1-mL monomeric avidin column. Retained proteins were eluted with an excess of biotin, and fractions of 500 μL were collected. Elution of the FCBP was assessed by ^{3}H-FC binding activity (hatched bars) and by immunoblotting (inset). For immunoblotting, the eluted fractions were transferred to a nitrocellulose membrane after lyophilization and SDS-PAGE. The blot was probed with the BMH1 antibodies, and immunodetection was performed by ECL.

not shown). When immunoblotted using the BMH1 antibodies in combination with the enhanced chemiluminescence (ECL) method (which is much more sensitive than silver staining), the 30- and 31-kD bands were clearly visible (inset, Figure 5). Note that the density of the immunodecorated bands in each fraction correlates well with the ^{3}H-FC binding activity of each fraction. In the control experiment, FCBP charged with FC was applied to the avidin column. Neither ^{3}H-FC binding activity nor immunodecorated bands were detected in the eluted fractions (data not shown).

Molecular Mass of the Native FCBP

Most 14-3-3 proteins are found as dimers in their native state. We tested whether the FCBP shares this property with other 14-3-3 proteins. To this end, we ran a purified FCBP sample (~1 μg of protein, as depicted in Figure 2C) over a high-resolution Superdex-200 column. We measured the ^{3}H-FC binding activity in the eluted fractions and immunoblotted the lyophilized fractions with the BMH1 antibodies (Figure 6). In each fraction, the ^{3}H-FC binding activity correlated well with the presence of the two antibody-detectable FCBP subunits. Both the peak of binding activity and the peak of immunodecorated bands were found at 70 ± 15 kD.

FC-Induced Recruitment of the FCBP

Infection of barley leaves with the powdery mildew fungus *Erysiphe graminis* induces a gene encoding a 14-3-3 protein homolog (Brandt et al., 1992). Could it be that the amount of FCBPs (after all, one of the best-characterized targets for fungal attack in plants) is also modified during fungal infection (i.e., during binding of FC) as suggested recently by Basel et al. (1994)? We addressed this question by in vivo incubation of oat roots with 10^{-5} M FC for different times (0, 5, 10, 20, 40, and 60 min). The duration of incubation in buffer was the same for all treatments (1 hr). After 1 hr, plasma membrane vesicles were isolated as described, and several tests were performed as given below.

For each time point, equal amounts of plasma membrane protein were separated on an SDS–polyacrylamide gel as shown by the silver-stained gel in Figure 7A. Equivalent gels were blotted and stained with the BMH1 antibodies (Figure 7B). Density scanning of the immunodecorated 30- and 31-kD bands showed a time-dependent increase in the amount of antibody-detectable FCBP in the plasma membrane (Figure 8A), with a rather short lag time that was less than 10 min. After 1 hr of incubation in FC, the increase amounted to ~70%; it must be noted that the incubation temperature was 16°C.

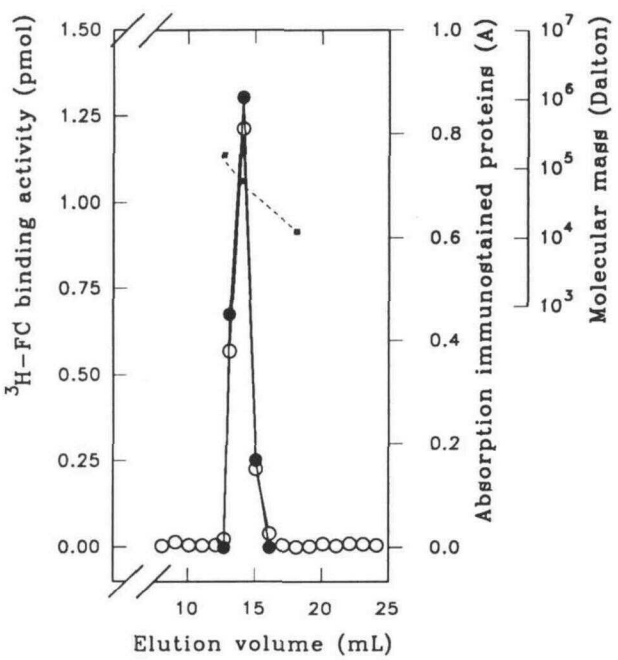

Figure 6. Determination of the Apparent Molecular Mass of the Native FCBP by Size Exclusion Chromatography.

The purified FCBP fraction (1 μg; Figure 2B, lane 3) was fractionated on a Superdex-200 size exclusion column. The column was calibrated with high molecular mass standards (dashed line). The location of the FCBP in the fractions (0.5 mL) was determined by means of ^{3}H-FC binding activity (open circles) and by immunoblotting (closed circles). For immunoblotting, the nitrocellulose blot was probed with the BMH1 antibodies and alkaline phosphatase–coupled secondary antibodies as the detection system. The amount of the immunostained polypeptides was quantitated densitometrically by laser scanning.

As a control, we also blotted and immunodecorated soluble cytoplasmic proteins, which were collected from the same roots at each time point (Figure 7C). The amount of cytoplasmic 14-3-3 homologs did not change during the FC incubation (Figure 8A), showing the specificity of FC action.

^{3}H-FC binding activity was detected at all six time points when a ^{3}H-FC binding assay was performed. The time-dependent increase in binding activity correlated very well with the increase in immunodecorated bands in the plasma membrane (Figure 8A).

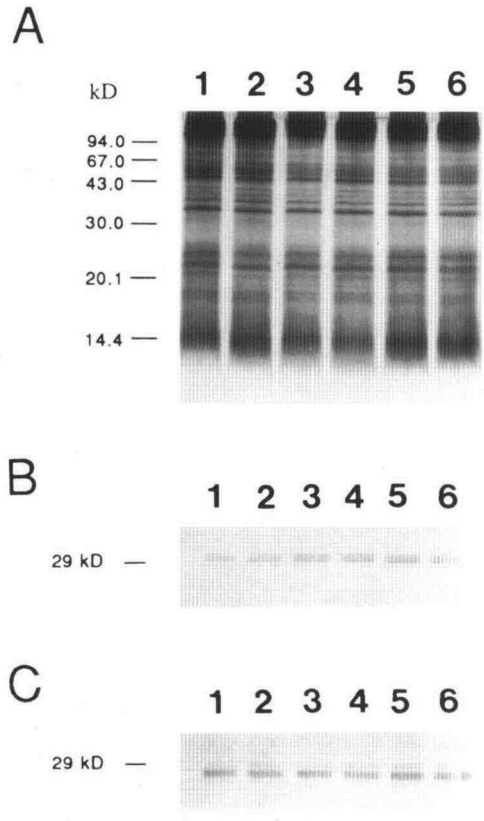

Figure 7. Immunodecoration of 14-3-3 Proteins after in Vivo Treatment with FC.

(A) SDS-PAGE of total plasma membrane proteins (7.5 μg per lane) from oat roots treated with 10^{-5} M FC in vivo for 0 (control, lane 1), 5 (lane 2), 10 (lane 3), 20 (lane 4), 40 (lane 5), and 60 (lane 6) min. The gel was silver stained. Numbers at the left indicate the positions of the molecular mass markers (in kilodaltons).

(B) Immunoblot of the gel as depicted in **(A).** The blot was probed with BMH1 antibodies and secondary antibodies coupled to alkaline phosphatase. The amount of the immunostained proteins was quantitated densitometrically by laser scanning. The position of the 29-kD molecular mass marker is given at the left.

(C) Immunoblot of cytoplasmic proteins (10 μg per lane) treated with FC as described in **(A).** The blot was probed, stained, and quantitated as described in **(B).** The position of the 29-kD molecular mass marker is given at the left.

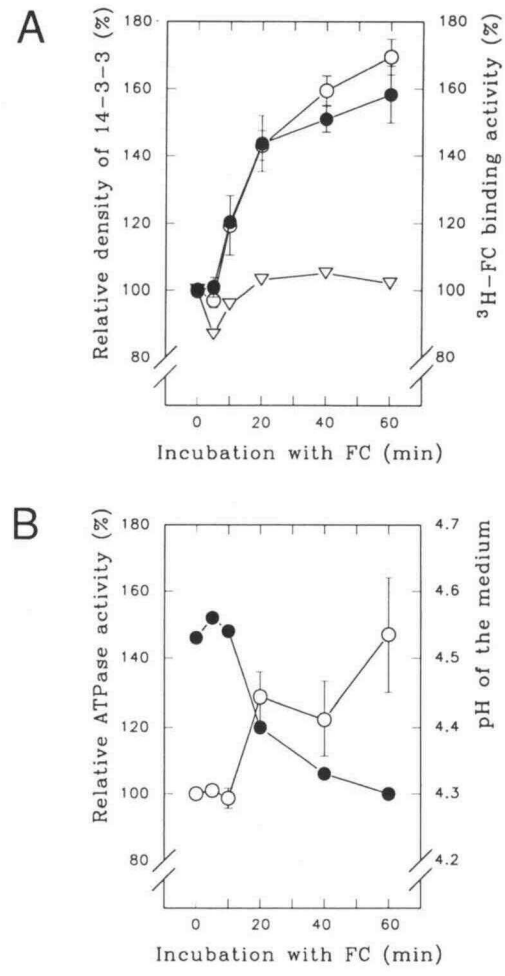

Figure 8. FCBP Accumulation, ^{3}H-FC Binding, and H^+-ATPase Activity in the Plasma Membrane after in Vivo Treatment with FC.

(A) Densitometric quantitation of the relative density of the immunostained proteins shown in Figure 7B (open circles; mean ± SD) and Figure 7C (inverted triangles) and the ^{3}H-FC binding activity of the plasma membrane fractions (closed circles; mean ± SD). The ^{3}H-FC binding activity in the control treatment was 26.6 pmol mg^{-1} protein, and the absorption of the immunostained proteins in the control treatment was 0.63 Arbitrary Units.

(B) Hydrolytic activity of the vanadate-sensitive, K^+-, and Mg^{2+}-dependent plasma membrane H^+-ATPase (open circle; mean ± SD) and the pH of the medium after 1 hr of incubation (closed circle). The H^+-ATPase activity in the control treatment (100%) was 0.730 μmol of Pi per mg $protein^{-1}$ min^{-1}.

Recently, Lanfermeyer and Prins (1994) reported on the modification of the plasma membrane–localized H^+-ATPase after in vivo treatment of oat roots with FC. When assayed at physiological pH (pH 7.2), we also observed an increase in hydrolytic activity of the H^+-ATPase in vitro (Figure 8B). It is interesting to note that the lag time of the FC-induced increase in H^+-ATPase activity is between 10 and 20 min, that is, the

increase in activity and amount of FCBP precedes the pump activation.

The pH of the medium was measured after the roots were incubated for 1 hr in buffer and FC for the time indicated (Figure 8B). The pH decreased with a lag time between 10 and 20 min, which correlates well with the lag time of pump activation.

DISCUSSION

FCBP Is a 14-3-3 Protein Homolog

In this study, we have shown that both the 30- and the 31-kD subunits of the FCBP belong to the highly conserved family of 14-3-3 brain protein homologs. Evidence that the FCBP is made up of two subunits of 30 and 31 kD was derived independently from FC–affinity purification experiments (de Boer et al., 1989; Korthout et al., 1994) and from photoaffinity labeling experiments using azido-labeled ^{3}H-FC (Feyerabend and Weiler, 1989; Oecking and Weiler, 1991). Amino acid sequence analysis of the 31-kD subunit, highly enriched in a purified FCBP fraction with high ^{3}H-FC binding activity (Figure 2), yielded the first indication of the relationship with the 14-3-3 protein family (Figure 3). Cross-reactivity of the two FC–affinity-purified FCBP subunits with BMH1 antibodies (Figure 5) demonstrated unambiguously that the conclusion derived from the sequence information was correct. This conclusion is further supported by the very good correlation between the presence of ^{3}H-FC binding activity and the intensity of immunodecorated 30- and 31-kD polypeptides in all experiments (Figures 5, 6, and 8). An alternative test would have been the immunoprecipitation of ^{3}H-FC binding activity with the BMH1 antibodies. However, these antibodies did not recognize the FCBP in native and active form (data not shown). This is not surprising because the FCBP is a membrane protein, and the antibodies were raised against soluble 14-3-3 homologs.

Aducci et al. (1993b) showed that in maize a 90-kD polypeptide copurifies with the 30-kD doublet and that both the 90- and the 30-kD doublet are photoaffinity labeled in the presence of azido-labeled ^{3}H-FC. In that article, the authors claim that under their conditions only the 90-kD protein is competent in binding ^{3}H-FC and that the 30-kD doublet may result from the breakdown of the 90-kD protein. Recent evidence shows that the 90-kD protein is not related to the 30-kD doublet (P. Aducci, personal communication). Although there is no 90-kD protein in our purified FCBP fractions, we will investigate the possibility that there is a second binding protein in the total plasma membrane fraction.

FCBP Is the First 14-3-3 Homolog with a Receptor Function

Recently, Ferl et al. (1994) presented the analyses of cDNA sequences of five Arabidopsis 14-3-3 homologs. From a phylogenetic analysis, they concluded that there has been an early and clear separation of the primary plant and animal 14-3-3 lineages. This agrees well with the fact that animals lack FC binding sites and with the conclusion from Meyer et al. (1993) that the FCBP has emerged very early in the evolution of land plants. Throughout evolution, 14-3-3 homologs have been highly conserved (Ferl et al., 1994), which may explain the very important function that the FCBP seems to have in plants: (1) all organs and developmental stages of the plant express the FCBP (Meyer et al., 1993); (2) expression of the FCBP is under developmental control (Basel and Cleland, 1992; Meyer et al., 1993); and (3) the structure and biochemical properties of the FCBP are highly conserved in different plant species (de Boer et al., 1989; Weiler et al., 1990; Oecking and Weiler, 1991).

One of the sequences from the oat FCBP (sequence 140 to 145; Figure 3) is in the center of a domain that is extremely well conserved in most 14-3-3 homologs (domain B; Ferl et al., 1994). The sequence of this region shows close similarity with the conserved C terminus of the family of Ca^{2+} and lipid binding proteins, the annexins. This region was shown to be involved in binding of protein kinase C (Mochly-Rosen et al., 1991). So, the FCBP seems to have the potential to bind a kinase C–type protein (see also de Boer et al., 1994). However, as pointed out by Ferl et al. (1994), because of the early separation of plant and animal lineages, we cannot assign beforehand a conserved, specialized biochemical function to plant isoforms based upon homology with a particular animal isoform with a known function. For the same reason, it is quite possible that 14-3-3 isoforms with a receptor-like function have evolved only in plants.

Subcellular Localization

The first report on the presence of FCBPs (Dohrman et al., 1977) mentions its localization in the plasma membrane, and it is now clear that the FCBP is an integral plasma membrane protein (Weiler et al., 1990). In this respect, the FCBP is thus unique among the known 14-3-3 proteins. Although the cytoplasmic protein fraction of oat roots clearly lacked ^{3}H-FC binding activity (data not shown), we expected to find cytoplasmic proteins related to the 14-3-3 protein family, considering the reports on 14-3-3 homologs in other plants (Brandt et al., 1992; de Vetten et al., 1992; Hirsch et al., 1992; Lu et al., 1992). Indeed, on protein gel blots with cytoplasmic proteins from oat root, the BMH1 antibodies cross-reacted with three polypeptides with apparent molecular masses of 27, 28, and 30.5 kD (Figure 4). It does not seem likely that these three polypeptides in the cytoplasm are precursors of active FCBP, because one would expect the precursors to be associated with endomembranes. In total cell extracts from Arabidopsis and maize, 14-3-3 antibodies also cross-reacted with at least five polypeptides (de Vetten et al., 1992; Lu et al., 1992).

Most 14-3-3 protein homologs are found as dimers in native form (Aitken et al., 1992), and the FCBP might be no exception.

The peak of the two immunodecorated FCBP subunits, eluted from the high-resolution size exclusion chromatography Superdex-200 column, ran at the same position as the BSA molecular mass marker. This is the same position at which the purified Arabidopsis GF14ω, expressed in *Escherichia coli* (monomer molecular mass of 32 kD when electrophoresed), elutes from a Superdex-75 column (Lu et al., 1994). In some experiments, a small peak containing high-affinity ^{3}H-FC binding activity and the immunoreactive doublet, of higher molecular mass (~500 kD) eluted from the column (data not shown). Currently, we are investigating whether this is a functional entity or an aggregation of proteins. It is interesting that with the radiation inactivation technique, a target size of the protein active in ^{3}H-FC binding of ~50 and 500 kD was determined (DeMichelis et al., 1989).

Ligand Control over the FCBP

Brandt et al. (1992) showed that infection of barley leaves with the powdery mildew fungus induces a gene encoding a 14-3-3 protein homolog. This up-regulation was not seen until a few hours after inoculation. Up-regulation of the FCBP by FC is much faster. Within 10 min, the amount of the antibody-detectable FCBP started to increase (Figures 7 and 8). The induction might occur at the level of transcription and/or translation because Basel et al. (1994) showed that cycloheximide partly inhibited the FC-induced increase in high-affinity ^{3}H-FC binding in the plasma membrane. The time course of the FC-induced increase of the antibody-detectable FCBP closely resembles that of the indoleacetic acid (IAA)-induced increase in plasma membrane H^+-ATPase levels in plasma membrane from maize, and it was shown that IAA acts at the level of transcription (Hager et al., 1991). However, FC had no effect on the amount of antibody-detectable plasma membrane H^+-ATPase (Hager et al., 1991). So, in combination with our observation that FC had no effect upon the amount of cytoplasmic 14-3-3 homologs either (Figure 7C), we concluded that the action of FC is rather specific.

In vivo–induced up-regulation of the FCBP clearly precedes the in vitro–measured increase in H^+-ATPase activity (Figure 8). This may explain a phenomenon observed by some groups, namely that modulation of the pump characteristics by FC is obvious only when the tissue is pretreated in vivo with FC (Johansson et al., 1993; Lanfermeyer and Prins, 1994). Is more FCBP indeed needed to effectively change the characteristics of the pump? Receptor up-regulation by binding of its own ligand is common in mammalian systems but has not been observed before in plants. Thus far, the physiological function of the observed FCBP up-regulation is not yet clear, and in this respect it will be very important to assess the biochemical properties of the induced 14-3-3 homolog in barley. If the latter protein is the FCBP, then the FCBP may play a wider role in the defense mechanism of plants against fungal attack.

The FC Signal Transduction Pathway

The most rapid cellular response to FC is a hyperpolarization of the membrane potential, with a lag time of less than 30 sec (Cleland et al., 1977). This FC effect has been ascribed to an activation of the primary H^+-pump, although the lag time of medium acidification is usually more than 5 min. Our observation that the modulation of the H^+-pump by FC takes at least 10 min (Figure 8) suggests that the fast response of the membrane potential may be a result of the modification of plasma membrane transport systems other than the H^+-pump, for example, K^+ channels (Blatt and Clint, 1989). Clearly, the FC signal transduction pathway is characterized by multiple responses, involving multiple targets with different time constants of (in)activation: (1) modification of secondary transport systems, (2) induction of FCBP recruitment into the plasma membrane, and (3) modulation of characteristics of the primary H^+-pump. A wide spectrum of cellular targets is typical for some protein kinases, such as protein kinase C (Hug and Sarre, 1993). Because several groups have shown that 14-3-3 homologs have the potential to act as regulators of protein kinase C (Aitken et al., 1992; Hirsch et al., 1992; Toker et al., 1992), we put forward a working model in which binding of FC to the FCBP modulates the activity of a protein kinase (see also de Boer et al., 1994).

If the FCBP has the capacity to bind a kinase (Mochly-Rosen et al., 1991; see previous discussion), then a polypeptide with kinase activity might, under certain conditions, copurify with the FCBP. Therefore, we are currently investigating the biochemical characteristics of a polypeptide with a molecular mass of ~67 kD that sometimes copurified in small quantities with the FCBP doublet using FC affinity chromatography (de Boer et al., 1989). For a number of reasons, this polypeptide is certainly not the FCBP because (1) photoaffinity labeling with azido-^{3}H-FC does not label any protein in this molecular mass range (Feyerabend and Weiler, 1989; Oecking and Weiler, 1991), and (2) the 67-kD polypeptide was often absent in purified FCBP fractions having a high activity of specific ^{3}H-FC binding (A.H. de Boer, unpublished results).

Traditionally, the mode of action of FC has been compared with that of auxin (Marrè, 1979). Although it is evident now that the IAA and FC signal transduction pathways are rather different, it may well be that they share a common element. This can be inferred from a number of independent observations: (1) phospholipase A_2, a G protein–coupled enzyme, induces the loss of high-affinity FC binding (Aducci et al., 1993a); (2) activation of G proteins results in the loss of high-affinity FC binding as well (de Boer et al., 1994); and (3) auxin activates phospholipase A_2, which is most likely mediated by G proteins, ultimately leading to kinase activation (Scherer and André, 1993). These observations could be reconciled by postulating a working model in which a kinase, which mediates the auxin signal and is under direct control of the FCBP, plays a pivotal role in both signal transduction pathways.

METHODS

Plant Material

Oat seedlings (*Avena sativa* cv Valiant) were grown on stainless steel screens over a 1 mM $CaSO_4$ solution at 25°C in the dark. Plasma membrane preparation was performed as described by de Boer et al. (1989). Briefly, 6-day-old roots were cut off and ground in ice-cold buffer containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 1 mM EDTA. The microsomal membranes and cytoplasmic proteins were isolated from the homogenate by filtration and differential centrifugation (see Figure 1). Plasma membrane vesicles were purified from microsomal membranes using the aqueous two-phase partitioning method described by Sandstrom et al. (1987). All steps were performed at 4°C.

Purification of the Fusicoccin Binding Protein

Plasma membrane proteins were solubilized from purified plasma membrane vesicles. Prior to solubilization, the plasma membrane vesicles were incubated with biotinylated fusicoccin (FC) (final concentration 10^{-6} M) in solution A (20 mM Tris-2-[*N*-morpholino]ethane sulfonic acid, pH 6.0, 20% [w/v] glycerol, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 2.3 mM DTT, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) for 2 hr at 30°C. After washing the biotinylated FC-charged vesicles (three times in solution A, 4°C), the vesicles were solubilized in solution B (20 mM Tris-HCl, pH 7.5, 20% [w/v] glycerol, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 150 mM NaCl, 2.3 mM DTT, 1 mM PMSF, and 1.4% [w/v] *n*-octyl β-D-glucopyranoside). The protein to detergent ratio was 1:20 (w/w), and the vesicles were sonified for 3 min at room temperature. After centrifugation, the solubilized plasma membrane proteins were dialyzed (Visking tube, cut off: 8000 D, 4°C) for 16 hr against solution C (20 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 2.3 mM DTT, 1 mM PMSF, and 0.2% [w/v] *n*-octyl β-D-glucopyranoside) to remove NaCl and glycerol and to lower the *n*-octyl β-D-glucopyranoside concentration from 1.4 to 0.2%. During dialysis, part of the *n*-octyl β-D-glucopyranoside–solubilized plasma membrane proteins precipitated (precipitated fraction), and these were separated by centrifugation from the plasma membrane proteins that remained in solution (low-detergent fraction). The plasma membrane proteins in the low-detergent fraction were applied to a Mono-Q HR 5/5 column (Pharmacia LKB, Uppsala, Sweden), equilibrated with 10 mM Tris-HCl, pH 7.5, and 5 mM sodium cholate, washed, and eluted with a KCl gradient from 0 to 600 mM, with a flow rate of 0.4 mL/min. Fractions of 1.5 mL were collected and screened for ^{3}H-FC binding activity. Fractions containing binding activity were pooled, lyophilized, and used for additional separation by SDS-PAGE.

Cytoplasmic proteins from oat roots were precipitated by adding solid $(NH_4)_2SO_4$ to give 80% saturation. After centrifugation (100,000*g*, 30 min), proteins were resuspended and desalted on a Sephadex PD-10 column (Pharmacia).

Size Exclusion Chromatography

Size exclusion chromatography was performed on a Superdex-200 HR 10/30 column (Pharmacia), calibrated with a gel filtration calibration kit (Pharmacia). The column was equilibrated in solution C. Approximately 1 μg of purified FCBP, obtained after the Mono-Q step, was separated on the column at a flow rate of 0.4 mL/min. Fractions of 0.5 mL were collected, screened for ^{3}H-FC binding activity, lyophilized, and analyzed by SDS-PAGE, which was followed by protein gel blotting.

Avidin Biotin Affinity Chromatography

FC affinity chromatography using biotinylated FC and monomeric avidin was performed as described by Korthout et al. (1994). Briefly, 15 mg of plasma membrane vesicles was charged with 10^{-6} M biotinylated FC in solution A as described above. After thoroughly washing the biotinylated FC-charged vesicles (three times in solution A, 4°C), the vesicles were solubilized and the soluble plasma membrane proteins were loaded on a 1-mL monomeric avidin column (Pierce, Rockford, IL), equilibrated in solution D (20 mM Tris- HCl, pH 7.5, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 150 mM NaCl, 2.3 mM DTT, 1 mM PMSF, and 1.0% [w/v] *n*-octyl β-D-glucopyranoside) at room temperature. At intervals of 45 min, 1-mL fractions were pumped into the column (a total of 15 fractions). After washing the column with 20 column volumes of solution D, the fusicoccin binding protein (FCBP)/biotinylated FC complex retained by the monomeric avidin was selectively eluted with 2 mM biotin in solution D at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected, screened for ^{3}H-FC binding activity, lyophilized, and analyzed by SDS-PAGE, which was followed by protein gel blotting.

In Vivo Treatment with FC

Six-day-old oat roots were harvested, washed in running tap water, and thoroughly mixed. From this mixture, six 100-g (fresh weight) samples were taken and stored on ice for 30 min. FC treatments were started for two samples at a time (starting with 5 and 10 min), and this was repeated for the other four treatments at 10-min intervals (20 and 40 min followed by 0 and 60 min). A 10-min FC treatment involved incubation in 150 mL of aerated 5 mM $CaSO_4$ solution for the first 50 min, and for the last 10 min, the $CaSO_4$ solution was replaced by a $CaSO_4$ solution containing 10^{-5} M FC. In the 0-min FC treatment, roots were incubated for 60 min in a $CaSO_4$ solution, and in the 60-min FC treatment, roots were incubated for 60 min in a $CaSO_4$ plus 10^{-5} M FC solution. The temperature of the incubation solutions was 16°C. After the 1-hr incubation period, roots were immediately ground in 200 mL of ice-cold buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 1 mM EDTA) using two blenders, and isolation of plasma membrane vesicles proceeded as described above. After the 1-hr incubation period, the pH of each solution was measured.

ATPase Assay

The plasma membrane–localized, vanadate-sensitive, K^+- and Mg^{2+}-dependent H^+-ATPase activity was measured as the release of Pi from ATP. Briefly, plasma membrane vesicles (5 μg per assay) were incubated in 0.5 mL of solution E (30 mM 3-[*N*-morpholino]propanesulfonic acid-KOH, pH 7.2, 5 mM $MgSO_4$, 50 mM KCl, 125 mM sucrose, 0.0125% [w/v] Triton X-100, 0.2 mM $(NH_4)_6Mo_{24}O_7$, and 5 mM Na_2ATP) and incubated at 30°C. After 15 min, the reaction was stopped with 1 mL of reagent according to LeBel et al. (1978). Color development was halted after 5 min by the addition of 0.1 mL of 34% sodium citrate. The amount of Pi released was determined spectrophotometrically at 750 nm. The addition of 0.5 mM Na_3VO_4 to the reaction assay completely inhibited the hydrolytic activity of the ATPases present.

Gel Electrophoresis

SDS-PAGE and protein gel blotting and electroelution were performed on the Bio-Rad Modular Mini Electrophoresis system. Proteins were analyzed by SDS-PAGE using a 15% acrylamide gel according to the method described by Laemmli (1970). Electrophoresis conditions were at 200 V for 1 hr. Proteins separated by SDS-PAGE were electroeluted from gel slices using a protein elution buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). Electroelution conditions were at 10 mA per gel slice at a constant current for 5 hr.

For two-dimensional gel electrophoresis, electroeluted proteins were separated by isoelectric focusing on tube gels containing 0.8% ampholines, pH 5 to 7, and 0.8% ampholines, pH 3.5 to 10, according to O'Farell et al. (1977). Isoelectric focusing conditions were at 375 V for 16.5 hr followed by 800 V for 1 hr. After isoelectric focusing, the tube gels were equilibrated in 62.5 mM Tris-HCl, pH 8.0, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol for 1 hr and then mounted on a 12.5% SDS-polyacrylamide gel for the second dimension as described above. Gels were stained with 0.2% Coomassie Brilliant Blue R 250 or with silver (Bio-Rad).

Protein Gel Blot Analysis

Proteins resolved by SDS-PAGE were transferred to nitrocellulose filters by electroblotting using a Tris-glycine buffer (Towbin et al., 1979); transfer conditions were 30 V, 40 mA overnight at room temperature. The blot was blocked with 2.5% (w/v) BSA in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20) for 1 hr at room temperature. Thereafter, the blot was probed for 1 hr (room temperature) with a polyclonal antibody (rabbit IgG) raised against the *BMH1* translation product, encoding a 14-3-3 homolog in *Saccharomyces cerevisiae* (BMH1 antibody). The blot was then washed five times with Tris-buffered saline and incubated for 1 hr with the secondary antibody (goat anti-rabbit–conjugated alkaline phosphatase or goat anti-rabbit–conjugated horseradish peroxidase). The two different detection systems that were used were the alkaline phosphatase system and the enhanced chemiluminescence system (ECL). For the alkaline phosphatase method, detection was performed with Western Blue color development substrate (Promega), which was used in combination with goat anti-rabbit–conjugated alkaline phosphatase as secondary antibody; the color density of the bands on the blots was quantitated densitometrically with laser scanning (Pharmacia LKB 2202 Ultroscan). With the ECL system, detection was performed using ECL protein gel blotting detection reagent from Amersham (Buckinghamshire, U.K.) in combination with goat anti-rabbit–conjugated horseradish peroxidase as secondary antibody. Chemiluminescence was visualized by short exposure (10 to 60 sec) to blue light–sensitive autoradiography film (Hyperfilm-ECL, Amersham). Biotinylated molecular mass markers were visualized with streptavidin-conjugated horseradish peroxidase and 4-chloronaphthol/3,3′-diaminobenzidine in stable peroxide substrate buffer (Pierce).

Protein and ^{3}H-FC Binding Assay

Protein concentrations were determined with bicinchoninic acid protein assay reagent (Pierce) using BSA as standard. Binding assays were performed with ^{3}H-FC (specific activity in May 1986, 35.2 Ci mmol^{-1}) as described by Korthout et al. (1994). Assay conditions were 4×10^{-8} M ^{3}H-FC in solution C for 12 hr at room temperature.

Protein Sequence Analysis

Sequence analysis of the 31-kD polypeptide excised from SDS–polyacrylamide gel was performed with an automated sequencer (Model 477A, Applied Biosystems) by Eurosequence B.V., Groningen, The Netherlands. Tryptic digests were fractionated by reverse phase HPLC on a Nucleosil 10 C18 (2 × 150 mm) column (Machery-Nagel, Düren, Germany). Peptide peaks, which were collected from the HPLC column, were concentrated, and peptides were sequenced.

ACKNOWLEDGMENTS

We are much indebted to Paul Van Heusden (University of Leiden, The Netherlands) for his generous gift of the BMH1 antibodies. We thank Sjoukje Heimovaara-Dijkstra (University of Leiden [RUL]/Netherlands Organization for Applied Scientific Research [TNO]) for running the two-dimensional gel and Ruud Kraayenhof for comments on the manuscript. This work was supported by the Technical Science Foundation (S.T.W.).

Received July 11, 1994; accepted September 29, 1994.

REFERENCES

Aducci, P., Crosetti, G., Federico, R., and Ballio, A. (1980). Fusicoccin receptors. Evidence for endogenous ligand. Planta **148,** 208–210.

Aducci, P., Ballio, A., and Federico, R. (1982). Solubilisation of fusicoccin binding sites. In Plasmalemma and Tonoplast: Their Functions in the Plant Cell, D. Marmé, E. Marrè, and R. Hertel, eds (Amsterdam: Elsevier Biomedical Press B.V.), pp. 279–284.

Aducci, P., Ballio, A., Blein, J.-P., Fullone, M.R., Rossignol, M., and Scalla, R. (1988). Functional reconstitution of a proton-translocating system responsive to fusicoccin. Proc. Natl. Acad. Sci. USA **85,** 7849–7851.

Aducci, P., Ballio, A., Donini, V., Fogliano, V., Fullone, M.R., and Marra, M. (1993a). Phospholipase A_2 affects the activity of fusicoccin receptors. FEBS Lett. **320,** 173–176.

Aducci, P., Ballio, A., Fogliano, V., Fullone, M.R., Marra, M., and Proietti, N. (1993b). Purification and photoaffinity labeling of fusicoccin receptors from maize. Eur. J. Biochem. **214,** 339–345.

Aitken, A., Collinge, D.B., Van Heusden, G.P.H., Isobe, T., Roseboom, P.H., Rosenfeld, G., and Soll, J. (1992). 14-3-3 Proteins: A highly conserved, widespread family of eukaryotic proteins. Trends Biochem. Sci. **17,** 498–501.

Assman, S.M., and Schwartz, A. (1992). Synergistic effect of light and fusicoccin on stomatal opening. Plant Physiol. **98,** 1349–1355.

Ballio, A., Chain, E.B., De Leo, P., Erlanger, B.F., Mauri, M., and Tonolo, A. (1964). Fusicoccin: A new wilting toxin produced by *Fusicoccum amygdali* Del. Nature **203,** 297.

Basel, L.E., and Cleland, R.E. (1992). Comparison of developmental gradients for growth, ATPase, and fusicoccin-binding activity in mung bean hypocotyls. Plant Physiol. **98,** 827–834.

Basel, L.E., Zukowski, A.T., and Cleland, R.E. (1994). Modulation of fusicoccin-binding activity in mung bean (*Vigna radiata* L.) hypocotyls by tissue maturation and by fusicoccin. Plant Physiol. **104,** 691–697.

Blatt, M.R., and Clint, G.M. (1989). Mechanisms of fusicoccin action: Kinetic modification and inactivation of K^+ channels in guard cells. Planta **178,** 509–523.

Brandt, J., Thordal-Christensen, H., Vad, K., Gregersen, P., and Collinge, D.B. (1992). A pathogen-induced gene of barley encodes a protein showing high similarity to a protein kinase regulator. Plant J. **2,** 815–820.

Cleland, R.E., Prins, H.B.A., Harper, J.R., and Higinbotham, N. (1977). Rapid hormone-induced hyperpolarization of the oat coleoptile transmembrane potential. Plant Physiol. **59,** 395–397.

de Boer, A.H., Watson, B.A., and Cleland, R.E. (1989). Purification and identification of the fusicoccin binding protein from oat root plasma membrane. Plant Physiol. **89,** 250–259.

de Boer, A.H., van der Molen, G.W., Prins, H.B.A., Korthout, H.A.A.J., and van der Hoeven, P.C.J. (1994). Aluminum fluoride and magnesium, activators of heterotrimeric G-proteins, affect high affinity binding of the fungal toxin fusicoccin to the fusicoccin binding protein in oat root plasma membrane. Eur. J. Biochem. **219,** 1023–1029.

DeMichelis, M., Pugliarello, M.C., Olivari, C., and Rasi-Caldogno, F. (1989). On the mechanism of FC-induced activation of the plasma membrane H^+-ATPase. In Plant Membrane Transport, J. Dainty, M.I. DeMichelis, E. Marrè, and F. Raso-Caldogno, eds (Amsterdam: Elsevier Science Publisher), pp. 373–378.

de Vetten, N.C., Lu, G., and Ferl, R.J. (1992). A maize protein associated with the G-box binding complex has homology to brain regulatory proteins. Plant Cell **4,** 1295–1307.

Dohrman, U., Hertel, R., Pesci, P., Coccuci, S., Marrè, E., Randuzzo, G., and Ballio, A. (1977). Localization of in vitro binding of the fungal toxin fusicoccin to plasma-membrane rich fractions from maize coleoptiles. Plant Sci. Lett. **9,** 291–299.

Ferl, R.J., Lu, G.H., and Bowen, B.W. (1994). Evolutionary implications of the family of 14-3-3 brain protein homologs in *Arabidopsis thaliana*. Genetica **92,** 129–138.

Feyerabend, M., and Weiler, E.W. (1989). Photoaffinity labeling and partial purification of the putative plant receptor for the fungal wilt-inducing toxin, fusicoccin. Planta **178,** 282–290.

Hager, A., Debus, G., Edel, H.-G., Stransky, H., and Serrano, R. (1991). Auxin induces exocytosis and the rapid synthesis of a high-turnover pool of plasma-membrane H^+-ATPase. Planta **185,** 527–537.

Hirsch, S., Aitken, A., Bertsch, U., and Soll, J. (1992). A plant homologue to mammalian brain 14-3-3 protein and protein kinase C inhibitor. FEBS Lett. **296,** 222–224.

Hug, H., and Sarre, T.F. (1993). Protein kinase C isoenzymes: Divergence in signal transduction? Biochem. J. **291,** 329–343.

Johansson, F., Sommarin, M., and Larsson, C. (1993). Fusicoccin activates the plasma membrane H^+-ATPase by a mechanism involving the C-terminal inhibitory domain. Plant Cell **5,** 321–327.

Korthout, H.A.A.J., van der Hoeven, P.C.J., Wagner, M.J., van Hunnik, E., and de Boer, A.H. (1994). Purification of the fusicoccin-binding protein from oat root plasma membrane by affinity chromatography with biotinylated fusicoccin. Plant Physiol. **105,** 1281–1288.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lanfermeyer, F.C., and Prins, H.B.A. (1994). Modulation of H^+-ATPase activity by fusicoccin in plasma membrane vesicles from oat (*Avena sativa* L.) roots. Plant Physiol. **104,** 1277–1285.

LeBel, D., Poinier, G.G., and Beaudoin, A.R. (1978). A convenient method for the ATPase assay. Anal. Biochem. **85,** 86–89.

Liss, H., Siebers, B., and Weiler, E.W. (1991). Characterization, functional reconstitution and activation by fusicoccin of a Ca^{2+}-ATPase from *Corydalis sempervirens* Pers. cell suspension cultures. Plant Cell Physiol. **32,** 1049–1056.

Lu, G., DeLisle, A.J., De Vetten, N.C., and Ferl, R.J. (1992). Brain proteins in plants: An Arabidopsis homolog to neurotransmitter pathway activators is part of a DNA binding complex. Proc. Natl. Acad. Sci. USA **89,** 11490–11494.

Lu, G., Sehnke, P.C., and Ferl, R.J. (1994). Phosphorylation and calcium binding properties of an Arabidopsis GF14 brain protein homolog. Plant Cell **6,** 501–510.

Marrè, E.A. (1979). Fusicoccin: A tool in plant physiology. Annu. Rev. Plant Physiol. **30,** 273–288.

Meyer, C., Waldkötter, K., Sprenger, A., Schlösser, U.G., Luther, M., and Weiler, E.W. (1993). Survey of the taxonomic and tissue distribution of microsomal binding sites for the non-host selective fungal phytotoxin, fusicoccin. Z. Naturforsch. **48c,** 595–602.

Mochly-Rosen, D., Khaner, H., Lopex, J., and Smith, B.L. (1991). Intracellular receptors for activated protein kinase C. J. Biol. Chem. **266,** 14866–14868.

Muromtsev, G.S., Voblikova, V.D., Kobrina, N.S., Koreneva, V.M., Sadovskaya, V.L., and Stolpakova, V.V. (1989). Fusicoccin in higher plants. Biochem. Physiol. Pflanzen **185,** 261–268.

Muromtsev, G.S., Voblikova, V.D., Kobrina, N.S., Koreneva, V.M., Krasnopolskaya, L.M., and Sadovskaya, V.L. (1994). Occurrence of fusicoccanes in plants and fungi. J. Plant Growth Regul. **13,** 39–49.

Oecking, C., and Weiler, E.W. (1991). Characterization and purification of the fusicoccin-binding complex from plasma membranes of *Commelina communis*. Eur. J. Biochem. **199,** 685–689.

O'Farell, P.Z., Goodman, H.M., and O'Farell, P.H. (1977). High-resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12,** 1133–1142.

Rasi-Caldogno, F., Pugliarello, M.C., Olivari, C., and DeMichelis, M.I. (1993). Controlled proteolysis mimics the effect of fusicoccin on the plasma membrane H^+-ATPase. Plant Physiol. **103,** 391–398.

Sandstrom, R.P., de Boer, A.H., Lomax, T.L., and Cleland, R.E. (1987). Latency of plasma membrane H^+-ATPase in vesicles isolated by aqueous phase partitioning. Plant Physiol. **85,** 693–698.

Schaller, G.E., Harmon, A.C., and Sussman, M.R. (1992). Characterization of a calcium- and lipid-dependent protein kinase associated with the plasma membrane of oat. Biochemistry **31,** 1721–1727.

Scherer, G.F.E., and André, B. (1993). Stimulation of phospholipase A_2 by auxin in microsomes from suspension cultured cells is receptor-mediated and influenced by nucleotides. Planta **191,** 515–523.

Toker, A., Sellers, L.A., Amess, B., Patel, Y., Harris, A., and Aitken, A. (1992). Multiple isoforms of a protein kinase C inhibitor (KCIP-1/14-3-3) from sheep brain. Eur. J. Biochem. **206,** 453–461.

Towbin, H., Staehelin, T., and Gordon, T. (1979). Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets. Proc. Natl. Acad. Sci. USA **76,** 4350–4354.

van Heusden, G.P.H., Wenzel, T.J., Lagendijk, E.L., Steensma, H.Y., and Van den Berg, J.A. (1992). Characterization of the yeast *BMH1* gene encoding a putative protein homologous to mammalian protein kinase II activators and protein kinase C inhibitors. FEBS Lett. **302,** 145–150.

Vera-Estrella, R., Barkla, B.J., Higgins, V.J., and Blumwald, E. (1994). Plant defense response to fungal pathogens. Plant Physiol. **104,** 209–215.

Weiler, E.W., Meyer, C., Oecking, C., Feyerabend, M., and Mithöfer, A. (1990). The fusicoccin receptor of higher plants. In Plant Gene Transfer, C.J. Lamb and R.N. Beachy, eds (New York: Wiley-Liss), pp. 153–164.