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Receptors for Plant Growth Regulators: Recent Advances

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Abstract. We have reviewed recent progress in research on plant growth regulator (PGR) receptors. For some growth regulators, no receptor protein has yet been identified, but promising new approaches are discussed. For other receptors, specific and sensitive probes have been developed and, in one case, the membrane-associated auxin-binding protein of maize, these have been used to study the function of the receptor. The maize receptor has been cloned and sequenced; cDNA probes will allow the expression of receptor genes in normal and transformed plants to be studied. PGR sensitivity mutants have been described and, in conjunction with biochemical probes, should prove valuable in elucidating the functions of receptors and the nature of subsequent signal transduction events.

In 1986 Professor M. Bopp wrote in the preface to the Heidelberg Conference proceedings that "hormone receptor substances are . . . not the subject of active research at the moment . . ." (Bopp 1986). This statement may have reflected the number of published reports appearing at the time, but research activity on receptors was on the increase and this is reflected in the progress we are able to review. The general area of plant growth regulator (PGR) receptors has been thoroughly reviewed by Venis (1985), Hall (1986), Libbenga et al. (1986), and Stoddart (1986), and so we will restrict ourselves here to developments in the last few years.

Most publications dealing with PGR binding use the terms PGR-binding site or binding protein rather than receptor and this needs to be explained. A receptor, in addition to binding a ligand in a specific and saturable manner, should generate some defined response as a consequence of that binding. Until recently, no PGR-induced response could be shown to be dependent on PGR binding and so most binding sites remain putative receptors. In some cases, experimental strategy is designed to increase understanding of binding sites regardless of their function, in the expectation that probes developed for these sites may then be applied to study genuine receptors (e.g., Brinegar et al. 1985, 1988, Smith et al. 1987). This review will show that several putative receptor polypeptides have now been identified and purified. In one case (see Auxin section) a range of specific probes has been raised, and these are being used to investigate the function of the protein. Only when we know the function of binding proteins will we be able to reflect on whether or not they are receptors. It is, therefore, prudent to use the terms "binding site" and "binding protein" until it can be shown that a site does transduce a stimulus into a response.

The review has been divided into sections dealing with binding sites for particular classes of PGR. There is no section for abscisic acid (ABA) because there have been no developments reported from Weiler's laboratory (or elsewhere) since the remarkable paper of Hornberg and Weiler (1984). However, the identification, in wheat, of an ABA-regulated promoter sequence that is expressed and regulated when incorporated into rice protoplasts, could provide a means of tracing back to the ABA receptor (Marcotte et al. 1988).

Ethylene

A very detailed review documenting the development of ethylene-binding site research has been written by Sisler (1990a) and other reviews have also appeared recently (Hall 1986, Hall et al. 1990).

Sisler has continued to define the binding site in terms of its ligand specificity (Sisler and Wood 1988a). A range of compounds was found to compete for binding of ^{14}C-ethylene without inducing a response (e.g., 1,3-butadiene); however, of those

examined, one compound (furan) both competed and induced a response. Carbon dioxide inhibits ethylene action, but competition for binding has not been shown convincingly and there is no inhibition of ethylene binding at all in vitro (Hall et al. 1987, Sisler and Wood 1988b). The interaction of ethylene and carbon dioxide is discussed in Sisler and Wood (1988b). The possibility that ethylene metabolizing enzymes might also function as receptors has been discussed (Hall et al. 1987, Sanders et al. 1986, Smith et al. 1987), although recent evidence indicates that ethylene metabolism is not linked to its mode of action (Sanders et al. 1989). A model is proposed in which ethylene oxide interacts with the binding site of the ethylene receptor and modulates the response induced by ethylene.

From measurements of ethylene binding in mung bean sprouts, three different binding sites have been distinguished by their dissociation rates (Sisler 1990b, Sisler and Wood 1987). The site with the shortest half-time (2–15 min) is lost on tissue homogenization, and ethylene bound to the site with an intermediate half-time (1 h) is also lost rapidly after homogenization. The site with the longest half-time (50 h) is also present in mung bean seeds and resembles the well-characterized membrane-bound site of *Phaseolus vulgaris* cotyledons (e.g., Hall et al. 1990). The very long dissociation time of this type of ethylene binding has facilitated purification and radiolabeled ethylene bound in vivo has been used to locate the binding protein. Potential photoaffinity labels have been reported (Quinn and Yang 1988, Sisler and Blankenship 1989), but so far only as abstracts to meetings. Purification has been hampered by the hydrophobicity of the protein, but Hall et al. (1990) report 162-fold purification by anion exchange chromatography (DEAE Sepharose and FPLC Mono Q) following a high pH membrane wash to remove peripheral membrane proteins and Triton solubilization. Residual phytohemagglutinin was removed on an immunoaffinity column to leave a single band after two-dimensional (2D) electrophoresis. Apparently, there is evidence that the binding site is a copper-containing glycoprotein.

Ethylene has no known physiological role in *Phaseolus* cotyledons, but Hall et al. (1990) have proposed using antibodies raised against the *Phaseolus* binding protein to study ethylene sites in pea. Peas show a number of rapid responses to ethylene (Warner and Leopold 1971), and a binding site with complementary properties has been identified (Hall et al. 1990). However, the site is not sufficiently abundant for purification without an affinity chromatography step.

Two other ethylene-responsive tissues which also bind ethylene are apple fruit and morning glory flowers. Both tissues undergo marked changes in sensitivity to ethylene; however no parallel changes in total ethylene binding have been found (Blankenship and Sisler 1989).

Ethylene-insensitive mutants of *Arabidopsis thaliana* have been found (Bleecker et al. 1988, M. A. Hall, personal communication) and have shown marked reduction in ethylene binding (Hall et al., unpublished data). These mutants have great potential for helping to elucidate the properties of ethylene receptors.

Gibberellin

Gibberellin (GA) receptors have been previously reviewed by Venis (1985) and Stoddart (1986). Some structural selectivity of a soluble, cytoplasmic GA-binding site has been demonstrated (Yalpani and Srivastava 1985), but there has been little progress with purification (Srivastava 1987). Binding of GA to a soluble site from maize leaf sheaths has also been reported, but binding was essentially nonreversible and poor selectivity between active and inactive GAs was found (Keith and Rappaport 1987). Attempts to identify GA-binding proteins by photoaffinity labeling with an azido-GA_4 analog (Beale et al. 1986) have also been disappointing. Only 15% of total incorporated label could be competed off with unlabeled GA_4 and no clear polypeptide candidates could be identified by SDS-PAGE and fluorography (Hooley et al. 1990). A much more promising approach has been the development of anti-idiotype antisera. Raised by immunizing with monoclonal antibodies to a GA derivative (Knox et al. 1987) and screening for inhibition of GA binding to these monoclonals, the antisera agglutinated oat aleurone protoplasts and inhibited GA-induced α-amylase synthesis (Hooley et al. 1990). The results suggest that a GA receptor is accessible on the outside of the plasma membrane. Such sera may also be of use for receptor purification. Unfortunately, GA-Sepharose, which will also agglutinate protoplasts, suffers from a high level of nonspecific protein binding and cannot be used for purification (Hooley et al. 1990).

The recently described dwarf *D8* mutant of maize, a GA-nonresponding dominant mutation (Fujioka et al. 1988), seems to be a good candidate for a mutation in the GA receptor or signal transduction pathway and should be a valuable tool.

GA stimulates the appearance of a protein which binds to genomic DNA in rice (Ou-Lee et al. 1988). The protein is tissue specific and binds to a 5′ upstream region of an α-amylase gene. No GA binding by this protein was found in a preliminary study, and there are likely to be intermediate steps be-

tween GA binding and the binding of this protein to the α-amylase gene. It will be interesting to see whether there are similarities between the region of DNA to which this GA-inducible promoter binds and the promoter sequence which is ABA-regulated (Marcotte et al. 1988).

Cytokinins

A protein fraction from barley leaves partially purified on a cytokinin affinity column (Selivankina et al. 1982) stimulated homologous RNA polymerase activity in the presence of cytokinin (Selivankina et al. 1985a), whereas a wheat protein purified in the same way as the barley did not affect RNA synthesis in barley (Selivankina et al. 1985b). Some purification of a barley cytokinin-binding protein (CBP) has been achieved (Romanov et al. 1986). It binds *trans*-zeatin and has a M_r of about 40 kD. Further characterization (Romanov et al. 1988) showed that high salt concentrations enhanced binding. Zeatin binding was reversible, of high affinity ($K_d \sim 1.5 \times 10^{-8}$ M), and specific. Displacement of zeatin by other compounds approximately reflects their activities as cytokinins.

Fluorescent anticytokinins have been used very successfully to assay for CBPs in tobacco callus cells (Hamaguchi et al. 1985). Three binding sites were identified, two soluble and one ribosomal. Of the soluble sites, one was of high affinity ($K_d = 2 \times 10^{-8}$ M for zeatin) and showed selectivity for a range of compounds in accordance with their biological activity, as did the ribosomal site ($K_d = 7.8 \times 10^{-7}$ M for zeatin). The second soluble site had relatively low affinity for zeatin ($K_d = 1.2 \times 10^{-5}$ M). The fluorescence assays are quick and sensitive and should be useful probes for other CBPs. However, this work does not seem to have been taken further, nor confirmed with radiolabled probes.

The most thoroughly characterized CBPs are those found in wheat and other cereal embryos. Antiserum to CBP showed that the protein was primarily localized in protein bodies in the tissues surrounding the embryonic axis and cross-reacted with proteins from a number of other plants (Brinegar and Fox 1987). The antiserum precipitated both CBP (54 kD monomer on SDS-PAGE) and higher M_r proteins from in vitro translation products (Brinegar and Fox 1985); however, affinity-purified antibodies identified only the 54-kD band (Brinegar et al. 1985), suggesting no major posttranslational modifications. The protein accumulates in the embryo 2–4 weeks postanthesis and can represent 9% of the soluble protein. Brinegar et al. (1985) discussed CBP, not as a receptor, but in terms of a protein that sequesters cytokinins, thus regulating cytokinin concentrations. Similarities with the vicilin storage proteins of legumes were also noted.

A photoactive cytokinin (Keim and Fox 1980) has been used to identify a peptide presumed to form part of the CBP-binding site (Brinegar et al. 1988). The photoaffinity label was shown to be bound to a single histidine residue. The protein chemistry techniques and probes, including a tritiated photoactive cytokinin of high-specific activity (Cooper et al. 1988), developed by Fox and co-workers, should be of great value if applied to the study of other CBPs. It may also be possible to raise antibodies to the binding-site peptide in anticipation that they may recognize other cytokinin-binding sites, although some degree of tertiary structure recognition may also be necessary.

Fusicoccin

The fungal toxin fusicoccin (FC) does not occur in plants other than *via* infection, but since high-affinity FC-binding sites were found in the plasma membrane, it has been suggested that there may be an endogenous ligand for these sites. No endogenous FC-like ligand has yet been identified, but anti-FC affinity chromatography (Marra et al. 1988) and anti-FC monoclonal antibodies (mAbs) should hasten progress, although the very high specificity of the mAbs for FC may be a limitation (Feyerabend and Weiler 1987).

Specific, high-affinity FC-binding sites are found to be broadly similar in a number of tissues (Venis 1985) and are distinct from auxin-binding sites. However, until recently, low abundance and instability of the solubilized sites (Aducci et al. 1984) has hindered purification and identification of the FC-binding protein (FCBP). A novel radiolabeled FC alcohol (FCol) has been used to study FC binding in *Vicia faba* (Feyerabend and Weiler 1988) and *Arabidopsis thaliana* (Meyer et al. 1989). FC binding in *Arabidopsis* has also been studied by Stout (1988) in cultured cells using the established ^{3}H-dihydro FC as ligand. In both *Vicia* and *Arabidopsis*, two sets of binding sites were reported by Weiler's group, whereas Stout (1988) detected only a single site in *Arabidopsis*, of intermediate affinity (see Table 1). Likewise, De Michelis et al. (1989) also found a single class of FC-binding sites in *Raphanus* and suggested that apparent site heterogeneity might result from hydrolytic activity during extraction.

FCol binding co-purifies with plasma membrane markers on density gradients and polymer two-phase partitioning (Feyerabend and Weiler 1988, Meyer et al. 1989). Two-phase partitioning yields

Table 1. Properties of FC-binding sites.

Plant	pH optimum	$t_{1/2}$ association (min)	Temperature (°C)	Apparent K_d (M)	Ligand	Reference
Vicia	5.5–6.0	6	25	1. 5×10^{-9} 2. 2.8×10^{-8}	FCol	Feyerabend and Weiler (1988)
Arabidopsis	5.5–6.0	12	25	1. 1.5×10^{-9} 2. 4.2×10^{-8}	FCol	Meyer et al. (1989)
Arabidopsis	5.5–6.0	30	20	1.3×10^{-8}	Dihydro FC	Stout (1988)
Raphanus	NS	NS	25	1.8×10^{-9}	Dihydro FC	De Michelis et al. (1989)

NS, not specified.

plasma membrane vesicles predominantly (approximately 75%) right side out, and the authors have used this feature to determine the orientation of FCBP in *Vicia* vesicles (Feyerabend and Weiler 1988). Trypsin removed FCol binding almost completely, suggesting that the FCBP was on the outside of the plasma membrane, an observation supported by the use of impermeant FC-protein conjugates and by earlier data on protoplast agglutination by FC coupled to bovine serum albumin (Aducci et al. 1980). Similar trypsin digestion by de Boer et al. (1989) produced only about 20% loss of FC binding from oat root plasma membrane vesicles.

Incubation of plasma membrane-enriched vesicles with a photoactive FC identified both in *Arabidopsis* (Meyer et al. 1989) and *Vicia* (Feyerabend and Weiler 1989), one principal polypeptide with an M_r of 34 kD on SDS-PAGE. The binding was specific and could be inhibited with an excess of unlabeled FC before photolysis. Active FCBP has been solubilized from the membranes of these species using either Mega 9 or octylglucoside and partially purified by FPLC.

Octylglucoside has also been used for solubilizing FCBP from oat plasma membranes purified by phase partition (de Boer et al. 1989). FC binding was found to be slower after solubilization ($t_{1/2}$ of association at 30°C-approximately 40 min), contrary to the results of Ballio et al. (1980). Activity was increased 50% by 1 M ammonium sulfate. An FC-affinity column was used to obtain a fraction showing two principal polypeptides on SDS-PAGE with M_r values of approximately 29.7 and 31 kD, the latter being equivalent to the 34-kD band of Meyer et al. (1989). Elution was with either 8 M urea or with a gradient of increasing SDS concentration; no data for specific elution with FC were given. A high background of nonspecific protein binding to the column was observed. That FC binding could be assayed after elution with urea or SDS suggests that FCBP is robust as long as it is protected from phosphatases and glycosidases (Aducci et al. 1984) and sugar diol oxidation (Meyer et al. 1989). Densitometric scanning of gels suggested a stoichiometry of one 29.7-kD polypeptide to two 31-kD polypeptides to give a possible native complex of 92 kD, consistent with gel filtration data suggesting a native M_r of around 80 kD (Stout and Cleland 1980).

The plasma membrane H^+-ATPase does not interact directly with FC (Aducci et al. 1988, Blum et al. 1988a, Stout and Cleland 1980). The H^+-ATPase is stimulated by FC (Barbier-Brygoo et al. 1989, Blum et al. 1988a, Rasi-Caldogno and Pugliarello 1985) although not after solubilization (Aducci et al. 1988, Blum et al. 1988a). De Michelis et al. (1989) showed that the saturation profiles of FC binding and FC activation of H^+-ATPase are very similar, supporting the view that ATPase activation is mediated by FC binding to its receptor. Reconstitution into liposomes of a purified plasma membrane H^+-ATPase with a crude mixture of membrane proteins containing FC-binding activity illustrated that ATP, FC, and FCBP together increased ATPase activity (Aducci et al. 1988). The identification and purification of the FCBP should allow more instructive reconstitution experiments to be performed.

It should be noted that FC stimulation of K^+ uptake by guard cells appears not to be simply a consequence of enhanced H^+ extrusion, but to involve a more direct modulation of K^+ channel activity by FC (Blatt and Clint, 1989, Clint and Blatt 1989).

Auxin

Membrane-associated auxin-binding sites from maize coleoptiles have been extensively studied since high-affinity binding was first described by Hertel et al. (1972). A high degree of purification was reported for the auxin-binding protein (ABP) by Löbler and Klämbt (1985a) and since then several groups have established purification protocols, raised antibodies, and made progress toward understanding the function of this protein.

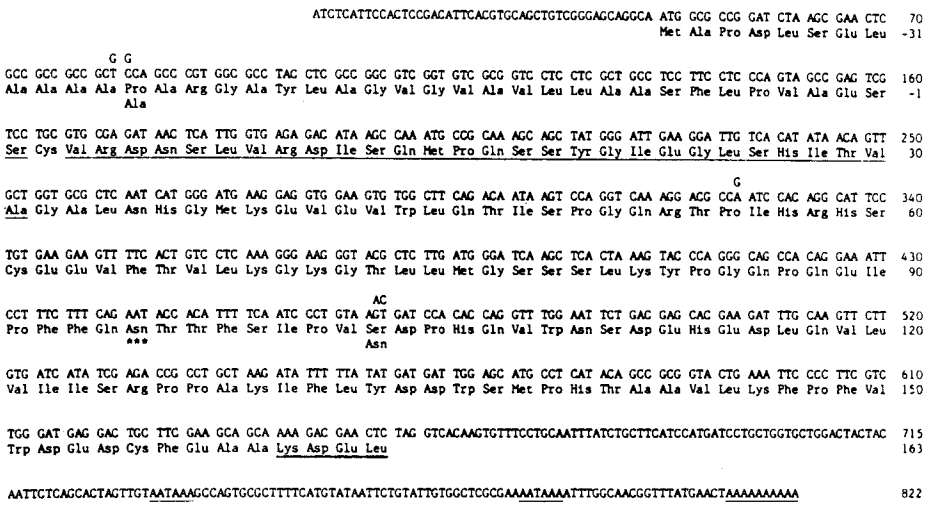

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the ABP cDNA in the cDNA clone (pAB420) carrying the longest insert. The different nucleotides and amino acids at various positions in nine cDNA clones are shown. Numbering of the amino acid sequence starts at the NH_2-terminal residue of the ABP isolated from maize shoots. The amino acid sequence determined by protein sequencing is underlined. Two polyadenylation signals and a poly(A) tract are also underlined. Asterisks indicate the potential *N*-glycosylation site. The consensus sequence necessary for retention in the ER lumen is marked by (=) (From Inohara et al. 1989).

Auxin-Sepharose chromatography and a series of immunoaffinity steps yielded a principal polypeptide with a M_r of 20 kD on SDS-PAGE (Löbler and Klämbt 1985a). Binding analysis of this protein showed that it had the characteristics of the widely studied high-affinity site I, with a sharp binding optimum at pH 5.5, K_d NAA = 5.7×10^{-8} M and comparable structural selectivity. ABP has a native M_r of 40–45 kD on gel filtration (see Venis 1985) and is likely, therefore, to be a dimer.

The identity of the 20-kD polypeptide has been confirmed by two other groups, each using different purification methods. Solubilized membrane proteins, passed through DEAE-Sephacel, were bound to an NAA-Sepharose 4B matrix and eluted with 10 mM NAA by Shimomura et al. (1986). A further step of gel filtration gives very pure ABP. This method has been adopted by Klämbt's laboratory (Barbier-Brygoo et al. 1989). Good purification has also been reported using conventional column chromatography, by anion exchange, gel filtration, and FPLC Mono Q (Napier et al. 1988). Pure ABP can be eluted after a further step of native gel electrophoresis. A second polypeptide, smaller by 1 kD, was found to co-purify with the major ABP, although eluting from anion exchange columns at a slightly lower salt concentration, and is thought to be a breakdown product of the ABP (Napier et al. 1988, Shimomura et al. 1986, 1988). Scatchard analysis of the purified ABP indicated only a single binding site and one site per dimer (Löbler and Klämbt 1985a, Shimomura et al. 1986), although this suggestion should be treated with caution because of the difficulties in estimating accurately the concentration of the pure protein.

Two oligonucleotides were synthesized on the basis of the N-terminal amino acid sequence of purified ABP (Inohara et al. 1989). Using a part-length cDNA clone to reprobe a cDNA library, they isolated a number of further clones including a full-length clone. Sequencing the clones has allowed them to deduce the full primary amino acid structure of ABP (Fig. 1). The mature (as extracted) protein is made up of 163 amino acid residues, while the sequence indicates a 38-residue signal peptide. The existence of a signal peptide for ABP had been predicted from in vitro translation experiments (Löbler et al. 1987). Analysis of the sequence shows that the only stretch of hydrophobic amino acids long enough to span membrane is in the signal peptide. The four C-terminal amino acids Lys-Asp-Glu-Leu probably account for the observations that the ABP is membrane-associated and located primarily within the endoplasmic reticulum (ER), for this sequence is shared by a number of proteins which are actively retained within the ER (Munro and Pelham 1987). There is one potential N-glycosylation site, consistent with the conclusion reached from endoglycosidase H digestion (Löbler et al. 1987, Napier et al. 1988). The ABP sequence shares no homology with any other protein, and the auxin-binding site has yet to be identified. Three other laboratories have obtained sequence data very similar to those of Inohara et al. (Hesse et al. 1989, Lazarus and Venis, unpublished results, Tillmann et al. 1989). Two minor ABP isoforms, together totalling <5% of the main ABP species, have been detected (Hesse et al. 1989).

Photoaffinity probes, such as 5-azido-[^{3}H]IAA (Jones et al. 1984, Jones and Venis 1989), together

with amino acid sequencing of labeled peptides could help to locate amino acids close to the binding site (see Brinegar et al. 1988). In membrane fractions of both zucchini (Hicks et al. 1989a) and tomato (Hicks et al. 1989b), this probe labeled specifically two polypeptides at 40 and 42 kD on SDS-PAGE. Labeling was not seen in membranes from shoots of the auxin-insensitive *diageotropica* mutant of tomato, although, in root membranes, labeling was comparable with the wild type. The relationship of these polypeptides to the 22-kD maize ABP remains to be evaluated.

The outer epidermis of maize coleoptiles is the site of auxin-mediated control of elongation (Kutschera et al. 1987). Immunofluorescence studies indicated that the ABP was localized preferentially within this layer of cells (Löbler and Klämbt 1985b) and binding assays of coleoptiles with and without the epidermis supported this suggestion (Shimomura et al. 1988). Correlations between the presence of ABP and auxin-responsiveness of tissues offer circumstantial evidence for the hypothesis that the ABP is an auxin receptor (Venis 1985, Jones et al. 1989). Much more substantial evidence comes from experiments in which ABP antibodies have been used to inhibit the action of auxin, presumably by inhibiting the correct functioning of the ABP. In the first such report, anti-ABP was found to inhibit auxin-induced curling of split coleoptiles and extension of coleoptile sections (Löbler and Klämbt 1985b). However, neither Shimomura et al. (1986) nor ourselves (unpublished observations) have been able to inhibit auxin responses of tissue sections using anti-ABP antibodies, which is consistent with data suggesting that molecules the size of immunoglobulins cannot pass through plant cell walls (Baron-Epel et al. 1988). On the other hand, it is difficult to explain the data of Löbler and Klämbt (1985b) if the antibodies could not reach the plasma membrane.

By using protoplasts such difficulties can be circumvented. Auxin-induced hyperpolarization of tobacco leaf protoplasts can be measured with microelectrodes (Ephritikhine et al. 1987). Monospecific ABP antibodies block their response (Barbier-Brygoo et al. 1989, see Fig. 2) without affecting FC-induced hyperpolarization (further demonstrating that auxin and FC receptors are distinct). Antiserum to a plasma membrane H^+-ATPase blocks both auxin- and FC-induced hyperpolarizations. The data suggest that the ABP is present on the outside of the plasma membrane, is tightly coupled to the activity of the H^+-ATPase, and is essential for auxin action. Additionally, the antibodies were raised against maize ABP and so the results suggest

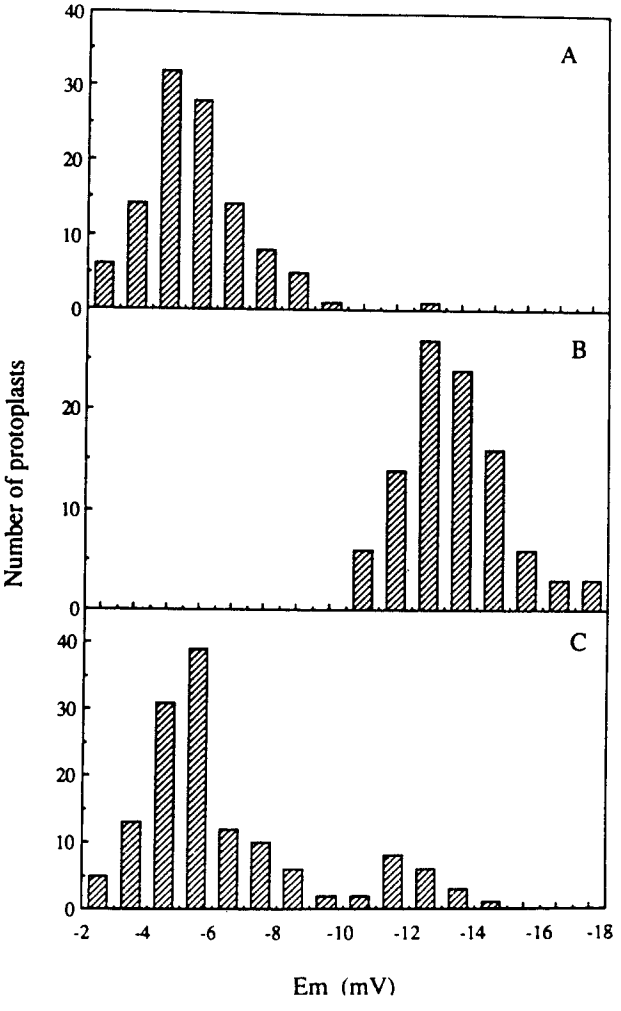

Fig. 2. The effects of ABP antiserum immunoglobulins on the NAA-induced transmembrane potential difference (Em) of tobacco protoplasts. The distribution of Em values is shown for (A) control protoplasts, (B) protoplasts in the presence of 5×10^{-6} M NAA, and (C) for protoplasts incubated with 4×10^{-7} M anti-ABP immunoglobulins for 2.5 h at room temperature before addition of 5×10^{-6} M NAA (Barbier-Brygoo et al. 1989).

that tobacco ABP shares some homology with maize ABP.

The roots of plants transformed by *Agrobacterium rhizogenes* are considerably more sensitive to auxin than untransformed roots (Shen et al. 1988). This differential sensitivity has been exploited in the tobacco protoplast system. By titrating ABP antibody against inhibition of auxin-induced hyperpolarization it was shown that with transformed cells relative to wild type, more antibody was needed to produce the same inhibition (Barbier-Brygoo et al. 1990) suggesting that increased abundance of ABP can be equated with increased sensitivity. Similarly, by adding exogenous ABP (from maize) to

protoplasts, auxin sensitivity was enhanced. The maize ABP must presumably be able to interact freely with the signal transduction pathway of tobacco.

All the electrophysiological data suggest that the ABP is an auxin receptor and further confirmation has come from the ability of our own polyclonal antibodies to block tobacco protoplast hyperpolarization (unpublished data). Evidence that functional auxin receptors are located on the exterior of the plasma membrane also comes from the ability of nonpermeant auxin analogs to evoke physiological responses (Venis and Napier 1990). Nevertheless, there is no doubt that the bulk of ABP—site I—is localized in the ER (see Venis 1985 for review of earlier work), and Shimomura et al. (1988) concluded that site I differs developmentally and immunologically from site II, which is thought to reside on the tonoplast and/or plasma membrane (Venis 1985). However, the electrophysiological evidence establishes clearly that antibodies raised by two different groups against bulk ABP can block an auxin response when the antibodies probably only have access to ABP on the outside of the plasma membrane. Therefore, tobacco plasma membrane and maize ER ABP *do* appear to be immunologically related, though whether or not they are identical remains to be established, as does their functional relationship.

The rapidity of the auxin-induced hyperpolarization (stable within 2 min, Ephritikhine et al. 1987) is at odds with membrane potential measurements of whole tissue in which there was depolarization before auxin-induced hyperpolarization, the latter starting 8–10 min after the addition of auxin (Bates and Goldsmith 1983, Felle 1988, Felle et al. 1986). The requirement for protein synthesis for auxin-induced proton secretion (Bates and Cleland 1979) also disagrees with the time-scale of hyperpolarization. It is possible that protoplasts are free from the intercellular electrical coupling which may modulate the response times measured in tissues. Such discrepancies need to be resolved. Clearly, however, the use of antibodies to inhibit specifically the function of the antigen, and perhaps the ability to add back exogenous antigen to restore activity, presents a very powerful experimental approach to expand our knowledge of how plant growth regulator receptors work.

As already indicated, the electrophysiological data imply homologies between maize and tobacco ABP. This is consistent with the ability of polyclonal antibodies to maize ABP to recognize homologous polypeptides in other species, including dicots, on Western blots (Venis and Napier 1990). Monoclonal antibodies to maize ABP have also been produced (Napier et al. 1988) and their epitopes partly mapped. Of particular interest are two mAbs that recognize epitopes within 1 kD of the C-terminus and which have been used to detect auxin-induced conformational change in the receptor (Venis and Napier 1990).

Nakamura and Ono (1988) have solubilized auxin-binding sites from membrane pellets of suspension-cultured tobacco cells. As with the sites described by Maan et al. (1983), auxin does not bind at 0–4°C and the very slow rate of association ($t_{1/2}$ = 15–20 min at 25°C) appears to rule out any direct relationship between this site and the ABP mediating tobacco protoplast membrane potential changes. The authors show also that it differs from the maize ABP discussed above. Root differentiation has been correlated with the presence of the tobacco ABP (Nakamura et al. 1988) but no causal relationship has been shown.

Auxin has been shown to stimulate NADH oxidase activity in plasma membrane fractions of soybean (Morré et al. 1986). Stimulation was two- to threefold and was maintained when the proteins were solubilized with detergent (Brightman et al. 1988). Purification, either by column chromatography or native PAGE, and subsequent SDS-PAGE identified three polypeptides that could be associated with activity (Brightman et al. 1988). The M_r values of these polypeptides did not correspond to the M_r of the maize ABP. It was not known if auxin bound to the oxidase complex or to any of the polypeptides. Stimulation of NADH oxidase activity on the plasma membrane would lead to cytoplasmic acidification, which has been observed in response to auxins (Felle et al. 1986) and further characterization of the interaction of auxin with the oxidase is awaited with interest.

Soluble auxin-binding proteins have also been reviewed recently (Libbenga et al. 1986, Venis 1985). The soluble ABP of suspension-cultured tobacco cells (Oostrom et al. 1980) has been partially purified on IAA–Sepharose (Mennes et al. 1987). Two of the proteins eluted were reported to have M_r values of about 50 kD on SDS-PAGE, about the same size as a labeled band obtained after phosphorylation with ^{32}P-ATP. The auxin eluate from the affinity column also stimulated total in vitro transcription by isolated nuclei, but the stimulation was extremely variable. Auxin induces changes in specific translatable mRNA species in tobacco (Van der Zaal et al. 1987) for which there are now cDNA probes (Mennes et al. 1990) and so it should be possible to test whether the soluble ABP can alter the level of these particular mRNAs. Heterologous

probes have also been developed for mRNAs that are rapidly upregulated by auxin (e.g., McClure et al. 1989).

Stimulation of total RNA synthesis by two soluble ABPs from mung beans (Sakai 1985, Sakai and Hanagata 1983) of relatively low affinity (K_d for IAA approximately 10^{-5} M) was independent of auxin (Sakai et al. 1986). Two classes of soluble ABP have been reported in cultured soybean cells (Herber et al. 1988, Jacobsen et al. 1987) using the techniques developed for pea epicotyl tissue (Jacobsen and Hajek 1985). The pea and soybean proteins have some similarities, but their high affinity for IAA suggests that they are different from the sites described by Sakai (1985). The soybean sites vary both in number and affinity through the cell cycle (Herber et al. 1988). The soluble ABPs from pea are reported to stimulate transcription in isolated pea nuclei in an auxin-dependent manner (Jacobsen et al. 1987), but no data were presented.

Phytotropins

The control of auxin transport is likely to be intimately associated with auxin action, and transport sites have been characterized at the physiological level. The transport protein, however, has yet to be identified. Phytotropins, which are structurally related inhibitors of auxin transport, show high affinity binding to microsomal membranes. They also stimulate auxin accumulation by sealed membrane vesicles by, it is presumed, blocking the auxin efflux carrier. Immunolocalization studies have suggested that the phytotropin-binding sites, used as a marker for the efflux carrier, are distributed preferentially at the basal ends of transporting cells. This and other earlier work have been reviewed previously (Venis 1985).

Specific naphthylphthalamic acid (NPA) binding has been used as a marker for plasma membranes (e.g., Lomax 1986). Lützelschwab et al. (1989) found that plasma membrane vesicles could be divided into two populations on the basis of their sedimentation in dextrose–sucrose gradients and characteristics of auxin accumulation. Both populations showed specific IAA accumulation but only the denser vesicles had high NPA binding and high phytotropin-stimulated IAA accumulation. The authors suggest that the two populations reflect vesicles from apical and basal (denser) parts of the cell. This would be compatible with a uniform distribution of uptake carriers and a basal distribution of efflux carriers (containing NPA-binding sites) in the cell, although other interpretations are possible. Phytotropin-stimulated uptake of auxin into vesicles can now be studied in maize (Heyn et al. 1987) and lupin (Sabater and Sabater 1986) in addition to zucchini.

Katekar (1987a) and Katekar et al. (1987a, b) point out that while phytotropins alter dramatically tropic responses, to concentrate solely on auxin transport inhibition as their mode of action could be misleading. In this context, it is of interest that two distinct sets of phytotropin-binding sites have been identified, of which only those of lower affinity appear to be directly related to the transport process (W. Michalke et al., submitted for publication). A detailed three-dimensional representation of the phytotropin-binding site has been constructed based on considerations of molecular conformation and electrostatic potential calculations (Katekar 1987b).

Monoclonal antibodies that may recognize the NPA-binding protein in peas (Jacobs and Gilbert 1983) did not stain maize or zucchini tissue, although they could partially block NPA binding and NPA inhibition of auxin transport in all three tissues (Jacobs and Short 1986). However, the only control for the inhibition experiments was the use of bovine serum albumin (BSA) in place of the antibody, so no definitive conclusions can be drawn from these observations. Immunoprecipitates of Triton-solubilized membranes gave a 77-kD band on SDS-PAGE again using only BSA as a control (Jacobs and Short 1986), and this was also seen in NPA-binding fractions separated by HPLC, along with two other bands (Jacobs and Short 1987). However, no further progress toward isolation and characterization of NPA-binding proteins has been reported. A photoactive phytotropin, the 5-azido analog of NPA, has been synthesized (Voet et al. 1987) and, if radiolabeled, may prove a useful probe.

Since phytotropins are synthetic PGRs, the question arises as to the normal function of the binding sites, and several laboratories are interested in the possibility that their presence may imply the existence of native phytotropins. Flavonoids are possible candidates for such a role, since Jacobs and Rubery (1988) have shown that certain flavonoids (e.g., quercetin) can compete with ^{3}H-NPA for binding and inhibit polar auxin transport. While their binding affinities are orders of magnitude lower than that of the high-affinity NPA site, this does not necessarily debar their physiological significance, especially in view of the observation that it is the lower affinity NPA site that is related to auxin transport (W. Michalke et al., submitted for publication). One apparent anomaly is that quercetin does not promote auxin accumulation by sealed vesicles from zucchini (G. F. Katekar, personal communication). It seems entirely possible that

other, higher affinity endogenous phytotropins with somewhat different properties may be found, by analogy with, for example, the different types of natural ligand found for benzodiazepine receptors (Sangameswaran et al. 1986).

Other Binding Interactions

Mention should be made of xyloglucan oligosaccharides that show anti-auxin activity (McDougall and Fry 1988). The structural specificity required for this activity suggests that a specific receptor may exist (McDougall and Fry 1989). If so, this must be distinct from the auxin receptor, since xyloglucan nonasaccharide does not compete for auxin-binding sites (Venis and Fry, unpublished data).

Structural specificity has often been used as an indication of the existence of a receptor protein. However, some structural specificity for growth regulators by synthetic lipid bilayers has been reported (Harkers et al. 1986, Stillwell and Hester 1984). In reports of the properties of artificial bilayers being altered by PGRs, few present control data for biologically inactive analogs (Bach 1986, Jones and Paleg 1988, Landau and Leshem 1988, Misso et al. 1986, Stillwell et al. 1987, 1988, 1989) and where both 2,4-D and IAA were used the two had conflicting effects (Schauf et al. 1987). The growth regulators generally need to be added at very high concentrations, typically about 10 mol % of the phospholipids or more, before having an effect, although Harkers et al. (1986) reported increasing K^+ leakage from vesicles between 10^{-6} to 10^{-3} M IAA and lipid oxidation may increase sensitivity (Misso et al. 1986).

A model embracing many of these reports proposed by Stillwell et al (1989) suggested that growth regulators act at defects in the lipid packing within the membrane and, for example, enhance permeability in consequence. Interestingly, the same paper shows that ABA is totally inactive when cholesterol is one of the constituents of the artificial membrane. Sterols, such as cholesterol, stabilize membranes, increase membrane order, and reduce permeability, probably by reducing the number of defects (Yeagle 1985) and so the significance in intact cells of the effects PGRs have on artifical membranes is uncertain. Evidence for an interaction between 2,4-D and plasma membrane proteins has been found (Morré et al. 1987), but once again no data for inactive analogues were presented.

Mutants

Mention has already been made of the potential uses of mutants with altered sensitivity to both ethylene and GA and there is no doubt that mutants have been underused in studying plant hormone action. Several other GA-response mutants have been described. The *slender* mutant of barley seems to allow tissues to express fully GA-induced processes independently of GA (Lanahan and Ho 1988), while ABA responsiveness is unchanged (Chandler 1988). A GA-response mutant in peas, *lv*, seems to enhance the plant's sensitivity to GA (Reid and Ross 1988), and in the GA-insensitive wheat mutant, Rht 3, IAA, low temperature, and phospholipid metabolism can all counter the effects of the mutation (Singh and Paleg, 1986).

Auxin-insensitive mutants have also been identified, for example, in *Arabidopsis* (Estelle and Somerville 1987), in barley (two mutants in which the lesion is associated with agravitropism; Tagliani et al. 1986) and the *diageotropica* mutant of tomato (Kelly and Bradford 1986).

It can be anticipated that the use of specific antibody and cDNA probes for receptors will identify some of these mutants with altered receptors or receptor abundance. Indeed, it has already been found that the shifted hyperpolarization–response curve of auxin-resistant tobacco protoplasts (Ephritikhine et al. 1987) can be mimicked by treating wild-type protoplasts with anti-ABP antibodies from maize—presumably, the result of reducing the endogenous population of functional receptors (Barbier-Brygoo et al. 1990). Clearly, the combination of biochemical technology and well-characterized mutants offers a very powerful approach to the study of receptor function.

Signal transduction

As growth regulator receptors are identified and their functions elucidated it is logical to start to consider the next steps in the signal transduction pathway. Considerable interest is being shown in the inositol polyphosphate pathway widely studied in animal cells (Sekar and Hokin 1986). Specific GTP-binding proteins have been identified in plants (e.g., Blum et al. 1988b, Jacobs et al. 1988) as has the phosphatidylinositol-specific phospholipase C (McMurray and Irvine 1988), and several reports have shown that inositol 1,4,5-trisphosphate (IP_3) is active on plant cell vesicles (e.g., Drøbak and Ferguson 1985, Schumaker and Sze 1987). The presence of a significant pool of IP_3 is less certain (Irvine et al. 1989, Rincón et al. 1989). It has been suggested that the auxin signal transduction pathway involves the IP_3 cascade (Ettlinger and Lehle 1988, Zbell and Walter-Back 1988), but these claims need further

substantiation. Recently, intriguing evidence has been obtained to suggest that in plants phosphatidylinositol phosphates themselves (Boss et al. 1990) and/or lysophospholipids (Scherer 1990) may act as second messengers, activating H^+-ATPases, and that their generation can be regulated by light and auxin, respectively.

In plants, no clear role for cyclic AMP has yet emerged, nor has a protein kinase C with characteristics of the mammalian enzymes been identified. Recently, oligonucleotides corresponding to a highly conserved region in the catalytic domain of animal protein kinases have been used to isolate corresponding plant genes. In these the putative catalytic domains most closely resemble the cyclic nucleotide-dependent protein kinases and protein kinase C family (Lawton et al. 1989). However, there were no homologies with mammalian regulatory domains, indicating that the plant homologues may be regulated by signals different from those used in animals.

Summary

The objective of all those engaged in receptor research is to understand how the growth regulator stimulus is transduced into observed physiological responses. It is, therefore, essential to elucidate the function of any protein that is shown to bind a growth regulator. In order to study function, specific probes for the protein are required, for which purified protein is usually needed. We have described the considerable progress made in purifying a number of binding proteins and in producing antibodies, photoaffinity ligands, and cDNA clones. The much more interesting task of using these probes to find out how proteins function is under way with exciting results already reported for the maize membrane-associated ABP. Promising advances have also been made in the techniques that can be used to identify and purify GA, ethylene, and cytokinin-binding proteins. Mutants will, undoubtedly, play an important role in growth regulator receptor research, and with an increased understanding of the receptors so, too, will our knowledge of the signal transduction pathways grow.

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