

AN ABSTRACT OF THE THESIS OF

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Plasma membrane (PM) vesicles were prepared from zucchini (*Cucurbita pepo*) hypocotyl tissue to high purity (approximately 95%). The vesicles accumulated a tritiated azido analog of indole-3-acetic acid (IAA), [<sup>3</sup>H]5N<sub>3</sub>-IAA (azido-IAA), in a manner similar to the accumulation of [<sup>3</sup>H]IAA. The association of the azido-IAA with the vesicles was saturable and subject to competition by various auxins. PM vesicles were incubated with azido-IAA and photolyzed with UV light (300nm) at -196°C resulting in the high specific activity labeling of a polypeptide doublet at 40 kDa and 42 kDa. The photolabeled polypeptides were of low abundance, and reduced labeling was observed in the presence of auxin competitors, indicating specific labeling. Furthermore, the pattern of inhibition was qualitatively similar to that observed for the uptake of [<sup>3</sup>H]IAA into PM vesicles. The protein doublet displays reduced labeling in hypocotyls but not roots of an auxin-insensitive mutant of tomato known as *diageotropica* (*dgt*) when compared to the parent, VFN8. Further characterization in zucchini PM preparations indicates that the azido-IAA binding proteins are

hydrophobic, integral membrane proteins that are enriched in the PM. They anomalously partition into the hydrophilic phase upon Triton X-114 extraction. This behavior has been suggested to be a general characteristic of proteins that associate to form multimeric hydrophilic channels. The proteins appear to associate as an 87 kDa dimer under native conditions, and two-dimensional electrophoresis indicates that the 40 kDa and 42 kDa polypeptides have isoelectric points of 8.2 and 7.2, respectively. Enzymatic digestion studies show that the two polypeptides of the doublet are closely related, and may represent modification states of the same protein. Tissue specificity experiments demonstrate that the polypeptides are photoaffinity labeled in auxin-responsive tissues such as hypocotyl and root but not in mature leaves. The functional identity of the 40 kDa and 42 kDa polypeptides is discussed, and they are compared to the 22 kDa auxin-binding protein from maize endoplasmic reticulum.

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# MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF TWO PLASMA MEMBRANE ASSOCIATED AUXIN-BINDING PROTEINS

## I. INTRODUCTION

Plant hormones play an essential role in controlling cellular events that are necessary for plant development. Among the five recognized classes of hormones, auxin (IAA, indole-3-acetic acid) is perhaps the most studied. Auxin has been implicated in a variety of developmental phenomena including apical dominance, leaf abscission, cell division, elongation, and differentiation. In spite of its involvement in such a diversity of plant responses, little is known concerning the molecular mechanisms of auxin action. However, based upon physiological evidence, several molecular models have been proposed that explain aspects of auxin-related development such as the specific polar transport of the hormone and auxin-induced cell growth. Plasma membrane (PM)-localized channels or receptors are widely accepted to be important components of these models. Using these models as a theoretical starting point, the goals of this thesis have been to directly identify auxin-specific channels or receptors in the PM and to gain insights into the role of these sites in plant development.

Several essential elements have made this approach feasible. The first is the methodology to produce highly purified PM vesicles having a "rightside-out" orientation which permits the study of PM components with minimal interference from other membrane sources. The other, more vital, element is the availability of a radiolabeled photoaffinity analog of auxin, [ $^3\text{H}$ ]5N $^3$ -IAA (azido-IAA). Azido-IAA has been demonstrated to have biological activity and transport characteristics similar to those of IAA. Thus, under the appropriate conditions, azido-IAA should covalently and radioactively label sites of auxin perception or action.

For this thesis, conditions were developed that permit the specific labeling of several PM proteins. Biochemical characterization and partial purification of these polypeptides reveal the properties of a auxin-specific receptor or channel. The physiological relevance of these proteins was also studied in an auxin insensitive mutant of tomato, *diageotropica* (*dgt*). This mutant is defective in the ability to perceive and grow in response to the hormone. The sum of this work is presented as a thesis in the form of three manuscripts.

## II. REVIEW OF LITERATURE

### Sites Associated with Auxin Transport

Auxins were the first phytohormones to be described and evidence of their existence was initially reported by Darwin (1880). His studies demonstrated that light is perceived at the tips of growing seedlings and that phototropic curvature is due to a basipetally transmitted and diffusible substance. This substance was isolated by Went (1928) and subsequently identified as indole-3-acetic acid (IAA). Auxins, of which IAA is the predominant native compound, are now recognized to be an important class of hormones involved in many aspects of plant growth and development. These include a variety of developmental phenomena such as apical dominance, leaf abscission, cell division, elongation, and differentiation (Davies, 1987). It has been widely accepted that IAA is synthesized from L-tryptophan in the apical meristem of shoots, in young leaves, and in fruits and flowers. The significance of L-tryptophan as a precursor is now in question, however, since the endogenous amount of IAA was found to be 50 times greater than normal in a tryptophan auxotroph of maize (Wright et al., 1991). Though auxin is synthesized at a limited number of sites, it is involved in the control of plant growth and development at sites

throughout the plant. Thus, auxin transport is an important area of study.

The transport of auxin through shoots is polar, since IAA moves only in a basipetal direction from the shoot apex (for review see Goldsmith, 1977). This is a facilitated transport process, since IAA moves at a nearly constant rate of 1 cm per hour. This velocity is more rapid than the diffusion of weak acids having pKas similar to that of IAA (Goldsmith, 1977). In wheat coleoptiles all cells may transport auxin (Perbal et al., 1982), in maize seedlings transport may be through the epidermis (Jones, 1990b), and in dicotyledenous species transport may be localized in cells surrounding the vascular tissues (Jacobs and Gilbert, 1983).

The chemiosmotic model of polar auxin transport was proposed by Rubery and Shelldrake (1974) to account for the observed characteristics of transport. According to this model, a pH gradient exists across the plasma membrane (PM) which is generated by an outwardly-directed  $H^+$ -ATPase. This results in a more acidic extracellular environment where the carboxyl group of IAA is protonated (IAAH) and electrically neutral. IAAH has increased membrane permeability relative to the anion ( $IAA^-$ ) and diffuses across the PM at a faster relative rate. In the more alkaline cytoplasmic environment, IAAH dissociates back to the less permeable  $IAA^-$  which accumulates within the cell. The polarity of transport is

thought to be derived from the secretion of IAA<sup>-</sup> via a selective anion carrier localized at the basal ends of cells involved in transport (Rubery and Sheldrake, 1974).

Since the original chemiosmotic model was proposed, evidence has accumulated that the uptake of IAA may be a carrier-mediated event. Jacobs and Hertel (1978) studied <sup>14</sup>C-IAA binding to PM fractions of *Cucurbita pepo* L. (zucchini). This binding was subsequently shown to be uptake of IAA into sealed vesicles (Hertel et al., 1983). That this is a saturable, carrier-mediated process was demonstrated by Lomax et al. (1985), who found that IAA accumulation into PM vesicles was greater than could be accounted for by diffusion gradients alone. Evidence has also been presented that the uptake carrier is electrogenic (Benning, 1986). Thus, uptake of IAA is hypothesized to occur via a specific IAA<sup>-</sup> / 2H<sup>+</sup> symport which is dependent on both electrical and pH gradients and is evenly distributed around the PM.

The efflux site has been proposed to be a nonelectrogenic site of IAA<sup>-</sup> secretion (Hertel, 1986). Evidence for the basal localization of this site was obtained by Jacobs and Gilbert (1983) using fluorescent monoclonal antibodies to a PM protein which specifically binds to the auxin efflux inhibitor, naphthylphthlamic acid (NPA). This approach resulted in the observation of immunofluorescent staining at the basal ends of pea stem cells within certain cell files associated with vascular

tissues. It should be noted, however, that the methodologies employed by Jacobs and Gilbert (1983) have been debated (Napier and Venis, 1990), so their conclusions should be evaluated with caution. By immunoprecipitation with the anti-NPA binding protein monoclonal antibodies, a 77 kDa protein, and several additional proteins, were detected by SDS-PAGE (Jacobs and Short, 1986, 1987). Approaches that do not involve the use of antibodies, such as the synthesis of an azido-NPA, have also been reported (Voet et al., 1987). More recently, Palme et al. (1991a) have reported the identification of a 30 kDa polypeptide with azido-NPA. NPA is a synthetic phytotropin, and some effort has been applied toward finding naturally occurring transport inhibitors. Flavonoids are possible candidates for these inhibitors, since it has been shown that certain flavonoids such as quercetin can compete with [<sup>3</sup>H]NPA for membrane binding (Jacobs and Rubery, 1988).



## Sites Associated with Auxin-Induced Growth

It has been hypothesized that auxins stimulate cell elongation through the action of a receptor-mediated and outwardly-directed proton secretion mechanism such as a  $H^+$ -ATPase located in the PM of target cells (Rayle and Cleland, 1970; Hager et al., 1971; Jacobs and Taiz, 1980). The secretion of protons is thought to acidify the cell wall which then leads to an increase in wall extensibility. This increase in wall plasticity then permits turgor-driven cell enlargement to occur. Several lines of evidence support this "acid growth" model. Treatment of auxin-responsive tissues with solutions of low pH stimulates growth (Rayle and Cleland, 1970). In addition, the specific application of auxin to coleoptiles of *Avena* (oat) was shown to result in acidification of the medium (Cleland, 1973; Rayle, 1973). The secretion of protons into the apoplast has subsequently been demonstrated in a number of studies (Cleland, 1976; Jacobs and Ray, 1976; Mentze et al., 1977; Evans and Vesper, 1980; Luthen et al., 1990). However, only recently has evidence been presented that proton secretion is due to an increase in the amount of  $H^+$ -ATPase at the PM (Hager et al., 1991). In this study, both the amount of detectable  $H^+$ -ATPase and the movement of membrane material from the endoplasmic reticulum (ER) to the PM of maize coleoptiles increased in response to IAA treatment. The increase in  $H^+$ -ATPase was immunologically detectable within

10 minutes of treatment and was interpreted as a precondition for growth. This and the fact that cordycepin reduced the amount of PM  $H^+$ -ATPase and growth indicate that the rapid induction of specific transcripts are an essential component of the initial responses to auxin (Hager et al., 1991).

A number of studies have examined the rapid expression (10-30 minutes) of mRNAs in response to auxin (for review see Hagen, 1989). Of these mRNAs, several (Walker and Key, 1982; McClure and Guilfoyle, 1987) have been shown to be most highly expressed in the elongating region of soybean hypocotyl. Utilizing the technique of tissue print hybridization, McClure and Guilfoyle (1989a, b) demonstrated that SAUR (Small Auxin Upregulated mRNA) transcripts were most abundantly expressed on the elongating side (lower) of gravistimulated soybean hypocotyls. SAUR transcripts were detected as early as 2.5 minutes after auxin treatment. Analysis of cloned cDNAs of these transcripts has revealed no homology to previously sequenced genes (Hagen, 1989).

The initial search for membrane-bound auxin receptors was begun by Hertel et al. (1972) and was based upon the binding of radiolabeled auxin to maize membrane fractions. This and later studies using maize and zucchini membranes (Dohrmann et al., 1978; Jacobs and Hertel, 1978) led to the identification of three different types of auxin binding sites. These sites were located at the ER (Site I),

tonoplast (Site II) and PM (Site III) and characterized as having different binding affinities for different auxins (Dohrmann et al., 1978; Jacobs and Hertel, 1978).

Since the initial characterization, dramatic progress has been made in the characterization of the ER binding protein from maize (for review see Jones, 1990a; Napier and Venis, 1990; Palme et al., 1991b). Several groups purified this protein as a 20 kDa to 22 kDa subunit from membranes of maize (Løebler and Klæmbt, 1985a; Shimomura et al., 1986). The purified protein was found to exist as a dimer of 40 kDa to 45 kDa under native conditions and bound auxin with an affinity, pH optimum and specificity similar to that of Site I (Løebler and Klæmbt, 1985a). Cloned cDNAs encoding the 22 kDa auxin-binding protein have been obtained by several independent groups utilizing somewhat different approaches (Hesse et al., 1989; Inohara et al., 1989; Tillmann et al., 1989). The derived amino acid sequence from each group was similar and predicted a 201 amino acid length protein containing a 38 amino acid signal sequence and a single N-linked glycosylation site. This glycosylation site was confirmed by enzymatic cleavage (Inohara et al., 1989) and chemical analysis (Hesse et al., 1989). The auxin-binding protein was also found to possess a carboxyl-terminal Lys-Asp-Glu-Leu (KDEL) sequence which has been shown to function as an ER retention signal (Munro and Pelham, 1987). No obvious membrane-spanning domains were reported as expected, since the protein was soluble without the aid of detergents. Several additional cDNAs were

reported which had differences in their deduced amino acid sequences suggesting the possibility of a gene family (Hesse et al., 1989).

There are several types of evidence which suggest that the 22 kDa ER protein is the receptor involved in auxin induced growth. Antibodies to the protein were reported to inhibit auxin induced growth in maize coleoptile sections (Løebler and Klæmbt, 1985b) However, Shimomura et al. (1986) reported that this result could not be reproduced, so the conclusions of Løebler and Klæmbt (1985a, b) may be inconclusive. Barbier-Brygoo et al. (1989) developed a system utilizing tobacco protoplasts in which auxin could be shown to induce hyperpolarization of the membrane. This response to auxin was blocked when antibodies to the 22 kDa ER protein or a yeast PM ATPase were added. This was interpreted as antibody binding at the PM surface (Barbier-Brygoo et al., 1989). The hyperpolarization response became more sensitive to auxin when purified 22 kDa auxin-binding protein was added (Barbier-Brygoo et al., 1990). In addition, protoplasts from auxin-resistant tobacco mutants were found to be less sensitive to auxin in this assay, whereas protoplasts from roots of plants transformed by *Agrobacterium rhizogenes*, which causes hairy root formation, were found to be 10-100 times more sensitive to auxin (Palme and Schell, 1991).

These results present a paradox in that the 22 kDa protein is located in the ER, yet antibodies to this protein apparently block auxin

induced hyperpolarization at the PM. Several authors have attempted to reconcile this inconsistency by proposing that the auxin-binding protein either cycles to the PM (Cross, 1991) or is secreted (Klæmbt, 1990). The protein may also function directly at the ER (Jones, 1990a). Several more trivial explanations have also been forwarded (Jones, 1990a).

A number of other auxin-binding proteins have been reported. Van der Linde et al. (1984) reported the preparation of a soluble fraction from tobacco callus that stimulated transcription in an IAA-dependent manner when added to purified tobacco nuclei. Sakai and Hanagata (1983) affinity purified a 390 kDa complex composed of 47 kDa and 15 kDa subunits. They also presented evidence that this preparation could stimulate transcription (Kikuchi et al., 1989). A soluble 25 kDa polypeptide was found to be specifically labelled by an azido-auxin in *Hyoscyamus muticus* suspension cells (McDonald et al., 1991). Prasad and Jones (1991) have utilized anti-idiotypic antibodies to identify a 65 kDa auxin-binding protein which is found in the nucleus and cytoplasm. Soluble sites have also been reported by other groups (for review see Napier and Venis, 1990). At this time the relationship of these various auxin-binding proteins is unknown.

### The *Diageotropica* Mutant of Tomato

The *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum*, Mill.) is a spontaneous variant of the parent variety, VFN8 (Zobel, 1969, 1972a). The predominant characteristics of the mutant are diageotropic shoot growth, lack of secondary vascular development, hyponastic leaves, and an inability to form lateral roots (Zobel, 1972a, 1974). Though the phenotype of *dgt* appears to be complex, it was found by Zobel (1972b) to behave as a single gene, recessive mutation. In addition to the original spontaneous mutant, a second spontaneous segregant and an ethylmethane sulfanate-induced mutant were recovered from the parent variety VF36 (Zobel, 1972b). Genetic studies indicated that the mutations in VF36 were allelic to those in VFN8 (Zobel, 1972b).

Zobel (1973) concluded that the primary effect of the *dgt* mutation was to reduce endogenous ethylene production compared to the VFN8. This was based upon the observation that the application of ethrel, a compound which is converted to ethylene gas at alkaline pH, at several concentrations appeared to normalize *dgt* plants with respect to leaf morphology, the formation of lateral roots and ageotropism (Zobel, 1973). His conclusion was also based on the direct measurement of ethylene production by the mutant in response to auxin. The *dgt*

mutant was found to produce only one sixth the amount of ethylene in response to auxin that VFN8 was capable of producing (Zobel, 1973).

Later, it was demonstrated that the ability of *dgt* plants to produce ethylene was unaffected, since other factors that caused ethylene production, such as anaerobiosis, normalized the *dgt* phenotype (Jackson, 1975, 1979). Bradford and Yang (1980) also demonstrated that ethylene production in response to stresses such as anaerobiosis and wounding was unaffected in *dgt*. Bradford and Yang (1980) also reported that *dgt* was highly insensitive to auxin with respect to ethylene production. If supplied with the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, however, the rate of ethylene production was comparable in *dgt* and VFN8. In more recent studies, it was determined that the primary defect associated with the *dgt* mutation is insensitivity to auxin (Kelly and Bradford, 1986). The mutant was shown not only to be defective in auxin-induced ethylene production but also to be hormone insensitive in terms of hypocotyl growth.

Daniel et al. (1989) examined both auxin transport and cellular growth in *dgt* and VFN8 seedlings. Polar auxin transport was measured and found to be elevated in capacity in *dgt*. In addition, the capacity for turgor-driven growth (osmotic potential) was greater in *dgt* than VFN8, while auxin-induced wall extensibility only occurred in VFN8 plants. A more recent confirmation of the inability of the mutant

to perceive auxin was reported by Clause and Rayle (1991). Utilizing oligonucleotide probes homologous to the auxin responsive SAUR transcripts (McClure and Guilfoyle, 1987), it was demonstrated that these transcripts were expressed in auxin treated hypocotyls of VFN8 but not in similarly treated hypocotyls of *dgt*.

Interestingly, heterozygotes have been reported to have a phenotype that is distinct from either *dgt* or VFN8. These plants display a "mottled" phenotype (Bradford, 1989) in which the small leaves display dark green sectors. In other respects, the heterozygotes are wildtype. For example, gravitropism, lateral root production and ethylene response to applied auxin are normal.



III. SPECIFIC PHOTOAFFINITY LABELING OF TWO  
PLASMA MEMBRANE POLYPEPTIDES WITH AN AZIDO AUXIN

## Abstract

Plasma membrane vesicles were isolated from zucchini (*Cucurbita pepo*) hypocotyl tissue by aqueous phase partitioning and assessed for homogeneity by the use of membrane-specific enzyme assays. The highly pure (ca. 95%) plasma membrane vesicles maintain a pH differential across the membrane and accumulate a tritiated azido analog of indole-3-acetic acid (IAA), [<sup>3</sup>H]5N<sub>3</sub>-IAA, in a manner similar to the accumulation of [<sup>3</sup>H]IAA. The association of the [<sup>3</sup>H]5N<sub>3</sub>-IAA with membrane vesicles is saturable and subject to competition by IAA and auxin analogs. Auxin-binding proteins were photoaffinity labeled by addition of [<sup>3</sup>H]5N<sub>3</sub>-IAA to plasma membrane vesicles prior to exposure to UV light (15 sec; 300 nm) and detected by subsequent NaDodSo<sub>4</sub>-PAGE and fluorography. When the reaction temperature was lowered to -196°C, high specific-activity labeling of a 40 kDa and a 42 kDa polypeptide was observed. Triton X-100 (0.1%) increased the specific activity of labeling and reduced background, which suggests that the labeled polypeptides are intrinsic membrane proteins. The labeled polypeptides are of low abundance as expected for auxin receptors. Furthermore, the addition of IAA and auxin analogs to the photoaffinity reaction mixture resulted in reduced labeling that was qualitatively similar to their effects on the accumulation of radiolabeled IAA in membrane vesicles. Collectively, these results suggest that the radiolabeled polypeptides are auxin receptors. The

covalent nature of the label should facilitate purification and further characterization of the receptors.

## Introduction

The plant hormone auxin (indole-3-acetic acid, IAA) plays an important role in a variety of developmental responses such as cell division, growth, and differentiation. While the molecular mechanism of auxin action is unknown, it is likely that auxin activates cellular responses by binding to specific receptor proteins (for reviews see Rubery, 1981; Cross, 1985; Libbenga and Mennes, 1987). We are particularly interested in the role of auxin-specific plasma membrane (PM) receptors in plant growth and development. At least three auxin-specific binding proteins are thought to be present in the PM; 1) an auxin uptake carrier (Rubery and Sheldrake, 1974; Hertel et al., 1983; Lomax et al., 1985; Benning, 1986; Lomax, 1986; Sabater and Rubery, 1987a,b), 2) an auxin efflux carrier (Hertel and Leopold, 1963; Goldsmith, 1977; Jacobs and Gilbert, 1983), and 3) an auxin receptor which is associated with an outwardly-directed proton pump thought to be involved in elongation growth (for review see Rubery, 1981; Libbenga and Mennes, 1987 and Discussion). The approach we are using to isolate and identify putative auxin receptors involves the preparation of highly-enriched PM vesicles by aqueous phase partitioning and photoaffinity labeling of polypeptides in those vesicles using the auxin analog, [ $^3\text{H}$ ]5N $_3$ -IAA (azido-IAA). Azido-IAA has auxin activity in several bioassays (Melhado et al., 1981) and exhibits a rate of polar transport similar to that of IAA (D.L.R. and T.L.L.,

unpublished data). The photoaffinity technique has advantages over radiolabeled auxins to study and isolate ligand-receptor complexes (Chowdhry and Westheimer, 1979). Foremost among these advantages is the ability of the azido-IAA to covalently link to its binding protein by photolysis, providing a stable chemical marker for the putative receptor proteins.

Exposure of azido-IAA to UV light (300 nm) results in photolysis of the aryl azide (azido group). The products of this reaction are N<sub>2</sub> gas and a highly reactive nitrene which, via a nucleophilic reaction would be expected to covalently label specific binding proteins. Using this compound, Jones et al. (1984a, b) were able to demonstrate quantitative differences in labeling of maize microsomal proteins in the presence of competing auxin or auxin analogs, although much background polypeptide labeling was evident (Jones et al., 1984b). Ripp et al. (1988) recently reported that a photoaffinity analog of sucrose labeled a 62 kDa membrane protein associated with sucrose transport in soybean. They found that lowering the temperature of the reaction mixture to -196°C (liquid nitrogen) resulted in reduced background labeling. Here we report that nonspecific azido-IAA labeling of membrane proteins can also be largely eliminated by conducting the photolysis at -196°C. Under these conditions, photolysis of zucchini (*Cucurbita pepo* L.) PM proteins with azido-IAA results in the high specific-activity labeling of a low abundance plasma membrane

polypeptide doublet which displays properties consistent with those expected for a plasma membrane auxin receptor.

## Materials and Methods

### Radiochemicals and Chemicals

[<sup>3</sup>H]indole-3-acetic acid, 22 Ci/mmol (1 Ci = 37 GBq), was purchased from Amersham, Arlington Heights IL; 7-[<sup>3</sup>H],5-azido indole-3-acetic acid (azido-IAA), 16 Ci/mmol, was synthesized as previously described (Jones et al., 1984b). All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. The ionophores (ION3) valinomycin, nigericin, and carbonylcyanide m-chlorophenylhydrazone, were prepared as a stock in 100% ethanol which contained 4  $\mu$ M of each compound (Sabater and Rubery, 1987a).

### Plant Material and Preparation of Plasma Membrane Vesicles

Seeds of zucchini squash (*Cucurbita pepo* L., cv. Dark Green, Ferry Morse Seed Co., Mountain View, CA) were sown in moist vermiculite and grown for 4 days in the dark at 28°C. Two cm hypocotyl sections were excised 5 mm below the apical hook, and homogenized in a Polytron (Brinkman Instruments, Burlingame, CA) (level 7-8) for 15 sec in 1 volume (1 ml/ g fresh weight) of ice cold Buffer I (0.25 M sucrose, 10 mM Tris/HCl, pH 7.5, 1 mM Na<sub>2</sub>EDTA, 1 mM dithiothreitol, 0.1 mM MgSO<sub>4</sub>, 0.2 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml pepstatin, and 1  $\mu$ g/ml leupeptin). This slurry was filtered through four layers of cheesecloth. The particulate material collected in the cheesecloth was homogenized again with an additional volume

of Buffer I for 15 sec. The combined filtrates were pooled and centrifuged at 4°C for 20 min at 3000x g (GPR H3.7 rotor, Beckman Instruments, Palo Alto, CA), and the pellets were discarded. The supernatant was centrifuged at 100,000x g for 40 min at 4°C (33,000 rpm, Ti50.2 rotor), and the resulting microsomal pellet was suspended in 2 ml of Buffer II (5 mM K<sub>2</sub>HPO<sub>4</sub> pH 7.8, 0.25 M sucrose, 4 mM KCl). Purified PM vesicles were then prepared by a version of the aqueous phase partitioning procedure recently reviewed by Larsson (1985). The resuspended microsomal vesicles were layered onto a phase separation medium containing 6.5% dextran, 6.5% PEG 4000, 0.25 M sucrose, 4 mM KCl, and 5 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.8. The mixture was degassed by vacuum evacuation for 20 min and centrifuged at 1000x g for 10 min at 4°C. The upper phase containing PM vesicles was extracted twice more, then removed, diluted with 4 volumes of Buffer I, and centrifuged at 100,000x g for 30 min. The resulting PM pellet was resuspended in approximately 0.5 ml of Buffer I, aliquoted, frozen in liquid N<sub>2</sub>, and stored at -80°C.

#### Marker Enzyme Analysis

Cytochrome c oxidase, a mitochondrial inner membrane marker (Tolbert, 1974), was assayed using freshly prepared PM vesicles. The membrane sample (20 µl) was mixed with 10 µl 3% digitonin in a cuvette and incubated 60 sec before the addition of 1 ml assay solution (0.2% reduced cytochrome c, 0.2 mg/ml EDTA, 40 mM potassium phosphate, pH 7.4). Cytochrome c was reduced with



sodium dithionate until A550/A565 was 9-10; excess reducing agent was removed by bubbling air through the solution. NADH-dependent cytochrome c reductase, an endoplasmic reticulum marker (Lord, 1983) was assayed in the same manner using an unreduced cytochrome c solution (0.27 mg/ml cytochrome c, 50 mM Tris/MES (morpholinoethanesulfonic acid) pH 7.5, 1mM KCN, and 0.1 mM NADH). Detergent-activated inosine diphosphatase (IDPase, Golgi marker) was assayed according to Nagahashi and Kane (1982) in the presence and absence of 0.03% digitonin. The nitrate-sensitive ATPase (a tonoplast marker), azide-sensitive ATPase (mitochondrial marker), and vanadate-sensitive ATPase (PM marker) were assayed as described by Sandstrom et al. (1987) except that 0.1 mM  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$  was included in the assay buffer.

#### [ $^3\text{H}$ ]IAA and [ $^3\text{H}$ ]5N<sub>3</sub>-IAA Association Curves

Association of labeled IAA with plasma membrane vesicles was assayed according to Benning (1986). Radiolabeled auxins were diluted in 10 mM disodium citrate/citric acid pH 5.5, 0.25 M sucrose, 5 mM  $\text{MgSO}_4$  to a final concentration of 4 nM. Tubes (1.5 ml) were prefilled with 0.9 ml of radiolabeled auxin (with or without the ionophore mixture, ION3, or unlabeled competitors) to which 1 g (fresh weight equivalent) of PM vesicles was added in 100  $\mu\text{l}$  of Buffer I. After 5 min, samples were centrifuged at 200,000x g for 5 min at 4°C (Beckman TLA 100.2 rotor) . The supernatants were discarded, and the radioactivity was extracted from the pelleted membranes with 0.5 ml of methanol.

The methanol extract was then added to Beckman Ready-Safe Scintillant for measurement of radioactivity.

### Photoaffinity Labeling

All manipulations were performed under red light. A quantity of PM vesicles equivalent to 100  $\mu\text{g}$  protein, as assayed by Coomassie brilliant blue binding (Spector, 1978), was diluted to 50  $\mu\text{l}$  with binding buffer (10 mM MES/1,3-bis[tris(hydroxymethyl)methylamino] propane pH 6.5, 0.25 M sucrose and 0.5  $\mu\text{M}$  [ $^3\text{H}$ ]-5N<sub>3</sub>-IAA). For competition experiments all competitors were added to a final concentration of 1 mM except 2,3,5-triiodobenzoic acid (TIBA) at 0.1 mM. Triton X-100, when present, was at 0.1%. Thirty seconds after addition of PM vesicles to the azido-IAA mixture, samples were transferred to glass cover slips which were placed on an aluminum block surrounded by liquid nitrogen (-196°C samples). After the samples were solidly frozen (1 min) they were irradiated for 15 sec with 300 nm UV light from a Fotodyne model 3-3000 transilluminator at a distance of 2.5 cm. Following irradiation, -196°C samples were stored overnight in the dark at -20°C. The vesicles were then washed by dilution into 1 ml of binding buffer and pelleted at 200,000x g for 5 min at 4°C. The pelleted PM vesicles were suspended in 20  $\mu\text{l}$  of NaDodSO<sub>4</sub> loading buffer and the PM proteins were separated by electrophoresis through 7.5-15% acrylamide gradient gels (Lomax et al., 1987), and stained with Coomassie brilliant blue (Hames, 1981) or silver (Heukeshoven and Dernick, 1985). Gels were soaked in a fluorographic enhancer

(Fluoro-hance, Research Products International) prior to drying and exposure to Kodak XAR-5 X-ray film for 5-10 days. For concentration of the proteins, the region corresponding to the labeled bands was excised and electroeluted according to Lomax et al. (1987).

## Results

### Phase-separated Zucchini Membrane Vesicles

Aqueous phase partitioning produces PM preparations from zucchini hypocotyl tissue that are substantially free of contaminating vacuolar, mitochondrial, endoplasmic reticulum, and Golgi membranes (Table III.1). Plasma membrane enrichment is indicated by the increase in  $\text{VO}_4$ -sensitive ATPase activity with an approximately five-fold reduction of the endoplasmic reticulum marker (cytochrome c reductase) and Golgi apparatus marker (IDPase) and greater than 100-fold reduction in marker enzyme activities for the tonoplast and mitochondria ( $\text{NO}_3^-$ -sensitive ATPase and  $\text{NaN}_3$ -sensitive ATPase, respectively). The purified vesicles are also enriched for pH-dependent IAA accumulation when compared to microsomes, indicating that the resulting PM preparations contain vesicles that are tightly sealed and able to maintain a pH gradient.

### Uptake of Azido-IAA into Membrane Vesicles

Previous data (Melhado et al., 1981) have shown that azido-IAA is an active auxin in several bioassays including polar transport in tobacco pith explants. However, prior to initiating attempts to photoaffinity label membrane proteins, we felt it important to verify that azido-IAA is accumulated in PM vesicles in a manner analogous

Table III.1. Marker enzyme analysis of microsome (M) and plasma membrane (PM) preparations.

<u>Ratio</u>	<u>Specific Activity*</u>		
	<u>M</u>	<u>PM</u>	<u>PM:M</u>
<u>Marker enzyme (organelle)+</u>			
VO <sub>4</sub> <sup>-</sup> -sens. ATPase (PM)	21.0	105.0	5.0
pH-dep. IAA transport (PM)	19.3	205.0	10.6
NO <sub>3</sub> <sup>-</sup> -sens. ATPase (Tp)	7.5	0.0	0
NaN <sub>3</sub> -sens. ATPase (Mt)	6.8	0.0	0
Cytochrome c oxidase (Mt)	34.7	0.5	0.01
Detergent-sens. IDPase (Golgi)	360.0	60.0	0.17
Cytochrome c reductase (ER)	156.6	30.2	0.19

+ Tp, tonoplast; Mt, mitochondria; ER, endoplasmic reticulum

\* Specific Activity, nmol per mg protein per min, except for pH dependent IAA transport, pmol IAA per mg protein per min.

to IAA and that it exhibits similar competition kinetics when compared with auxin analogs.

As shown in Table III.2, zucchini PM vesicles were able to accumulate both [ $^3\text{H}$ ]IAA and [ $^3\text{H}$ ]5N<sub>3</sub>-IAA in similar quantities in the presence of a pH gradient. Accumulation was reduced by the addition of ionophores (ION3) which dissipate both the pH gradient and any membrane potential which is present. Competition curves for both [ $^3\text{H}$ ]IAA and [ $^3\text{H}$ ]5N<sub>3</sub>-IAA were obtained using various synthetic auxins and auxin analogs, and the concentrations giving 50% inhibition of association (IC<sub>50</sub>) were calculated (Table III.3). For comparison, we have included values obtained for [ $^{14}\text{C}$ ]IAA uptake into zucchini microsomal membrane vesicles (Lomax, 1986). In each case, non-radioactively labeled IAA and 2-naphthaleneacetic acid competed well for association, whereas 1-naphthaleneacetic acid and benzoic acid were much less effective and 2,4-dichlorophenoxyacetic acid was intermediate in ability to compete. Collectively, these data indicate that azido-IAA and IAA are taken up into PM vesicles in a similar manner and that competitors of IAA association also alter azido-IAA association in a qualitatively similar fashion.

#### A Polypeptide Doublet is Labeled to High Specific Activity at -196°C

Initial attempts to photoaffinity label PM auxin receptors were performed at 0-4°C. After 300 nm UV irradiation followed by denaturing gel electrophoresis and fluorography, [ $^3\text{H}$ ]5N<sub>3</sub>-IAA was

Table III.2. Ionophore-sensitive association of [ $^3\text{H}$ ]IAA or [ $^3\text{H}$ ]5N $_3$ -IAA with zucchini PM vesicles.

	<u>radiolabel associated (cpm)</u>	
	<u>-ION3</u>	<u>+ION3</u>
[ $^3\text{H}$ ]5N $_3$ -IAA	4840	867
[ $^3\text{H}$ ]IAA	6668	815

The association of [ $^3\text{H}$ ]5N $_3$ -IAA or [ $^3\text{H}$ ]IAA (4 nM) with zucchini PM vesicles was measured by centrifugation assay in the presence and absence of the ionophore mixture, ION3 (4 $\mu\text{M}$ ).

Table III.3. Effect of auxin analogs on the inhibition of accumulation of radiolabeled IAA or azido-IAA.

radiolabeled IAA derivative	<u>IC<sub>50</sub> values</u>				
	IAA	2-NAA	2,4-D	1-NAA	BA
[ <sup>3</sup> H]5N <sub>3</sub> -IAA	7.3	7.3	6.2	5.7	4.7
[ <sup>3</sup> H]IAA	6.7	7.4	6.2	5.5	4.4
[ <sup>14</sup> C]IAA*	7.1	6.4	5.4	4.7	4.5

IC<sub>50</sub> values (-log of conc. giving 50% inhibition of accumulation) were calculated from experiments measuring the pH gradient-dependent (ION3-sensitive) accumulation of either [<sup>3</sup>H]5N<sub>3</sub>-IAA, [<sup>3</sup>H]IAA, or [<sup>14</sup>C]IAA in the presence of seven concentrations of auxin analog ranging from 10<sup>-8</sup> to 10<sup>-4</sup>M. Nonspecific radioligand association in the presence of 10<sup>-4</sup>M unlabeled IAA was 25% of the control value. NAA, naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, benzoic acid.

\* Taken for comparison from Lomax (1986), data generated using microsomal membrane preparations rather than PM.



found to be associated with more than ten polypeptides (Figure III.1). The intensity of polypeptide labeling under these conditions appeared to be roughly proportional to the stained quantity of protein (compare Figure III.1 to Coomassie-stained gel in Figure III.2b). These findings suggested that since azido-IAA photoaffinity labeling at 0-4°C was relatively nonspecific, the identification of putative auxin receptors would be problematical.

A different result was obtained when the temperature of the PM/azido-IAA mixture was lowered to -196°C prior to a 15 sec UV exposure. Under this condition fluorography revealed that labeling was limited to a polypeptide doublet of 40 kDa and 42 kDa (Figures III.2a and III.4). Since we could not visualize the doublet after either Coomassie or silver staining (Figures III.2b and III.2c), high specific activity labeling was indicated. After a nine-fold concentration of the gel region containing the doublet, polypeptide bands became visible by silver staining (Figure III.3). Addition of unlabeled IAA prior to irradiation reduced photoaffinity labeling by azido-IAA (Figure III.2a). Competition was more pronounced in the presence of Triton X-100 which both increased the intensity of labeling in the absence of unlabeled IAA and reduced the amount of background per lane. The enhancement effect of Triton X-100 is likely to be due to solubilization of extrinsic PM proteins which accounts for the reduced background in these lanes; this explanation would also account for the reduced quantity of visible protein in the matching Coomassie-stained gel lanes

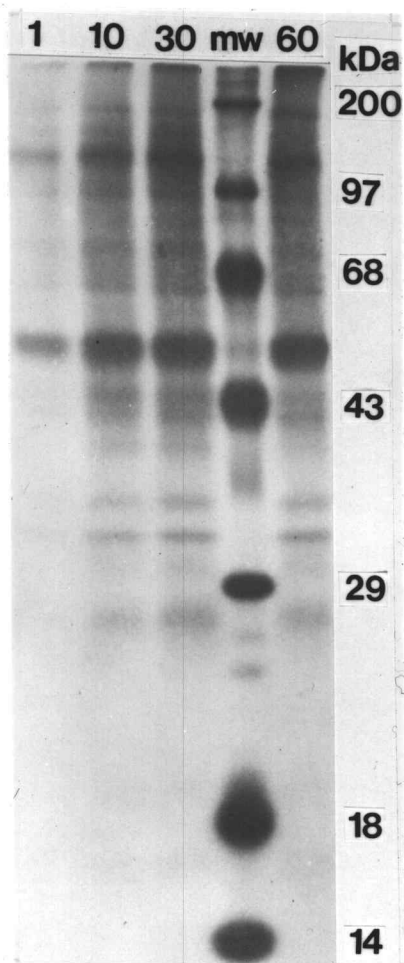


Figure III.1. Fluorograph showing nonspecific photoaffinity labeling at 0-4°C. Zucchini PM vesicles were irradiated with 300 nm UV light in the presence of 0.5  $\mu$ M [ $^3$ H]5N $_3$ -IAA for 1, 10, 30, or 60 sec. Each lane contains 100  $\mu$ g protein. The molecular weights of marker proteins (lane mw) are given at right.

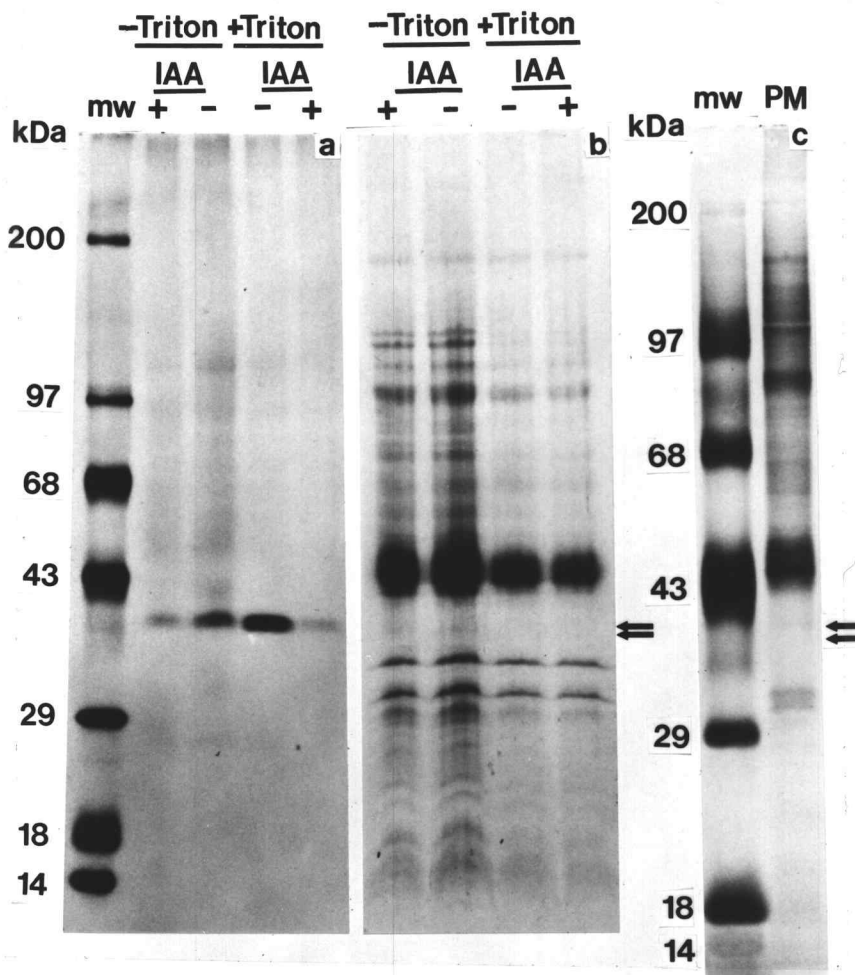


Figure III.2. High specific-activity photoaffinity labeling of zucchini PM proteins at  $-196^{\circ}\text{C}$ . Fluorograph (a) shows labeling with [ $^3\text{H}$ ]5N<sub>3</sub>-IAA ( $0.5\ \mu\text{M}$ ) in the absence (-) or presence (+) of  $1\ \text{mM}$  unlabeled IAA. The labeling was performed with and without the addition of  $0.1\%$  Triton X-100. The corresponding Coomassie-stained gel (b,  $100\ \mu\text{g}$  protein per lane) and a silver-stained gel of  $15\ \mu\text{g}$  PM protein (c) are shown for comparison. Arrows indicate positions of the  $40\ \text{kDa}$  and  $42\ \text{kDa}$  polypeptides estimated from alignment with the fluorograph. Molecular weights of marker proteins (mw) are given.

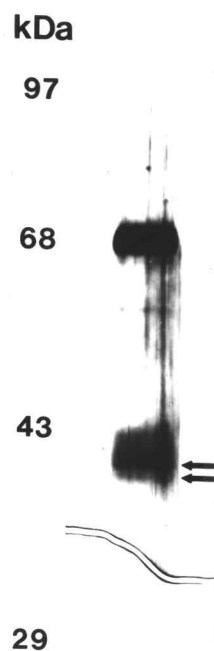


Figure III.3. Silver-stained gel demonstrating visualization of polypeptides following nine-fold enrichment by preparative electrophoresis and electroelution of the azido-IAA labeled region. Each band (arrows) is estimated to be about 50 ng protein. The 68 kD protein band is bovine serum albumin used to enhance protein precipitation.

(Figure III.2b) and suggests that the labeled 40 kDa and 42 kDa doublet is intrinsic in the PM.

The effect of varying the pH of the medium in which the PM vesicles were suspended on labeling of the polypeptide doublet was also investigated. The greatest amount of photoaffinity labeling by [<sup>3</sup>H]5N<sub>3</sub>-IAA was observed at pH 6.5 (Figure III.4). Significant fluorographic signals were also produced at pH 5.5, but medium with a pH of 7.5 resulted in a greatly diminished signal (Figure III.4).

#### Auxins that Compete for Uptake In Vitro Reduce Azido-IAA Labeling

When competitors of IAA and azido-IAA uptake were added to the photoaffinity reaction prior to UV irradiation, a pattern of inhibition emerged which is qualitatively similar to the effects of the analogs on uptake (compare Figure III.5 to Table III.3). IAA and 2-naphthaleneacetic acid largely prevented labeling, whereas 2,4-dichlorophenoxyacetic acid and 1-naphthaleneacetic acid had lesser effects. Addition of tryptophan, which is a presumed precursor of IAA and which does not compete for uptake in vitro had no effect on labeling (Figure III.5). Though the overall intensity of signal may be reduced by the addition of auxin analogs, the polypeptides of the doublet appear to label with equal intensity relative to each other regardless of the competitor present during the photoaffinity reaction. Addition of the auxin transport inhibitor TIBA to the azido-IAA photoaffinity reaction produced no significant reduction in labeling (Figure III.4).

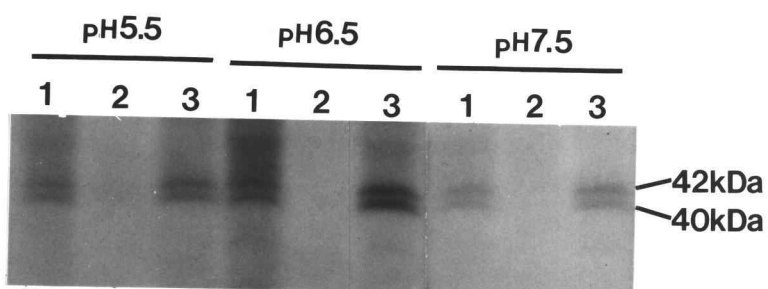


Figure III.4. Fluorograph showing the effect of pH {10 mM MES/1,3-bis[tris(hydroxymethyl)methylamino] propane} on azido-IAA labeling of the 40/42 kDa doublet (compare lane 1 at pH 5.5, 6.5, and 7.5). Also shown is the effect of 1 mM IAA (lane 2) and 0.1 mM TIBA (lane 3) at each pH. Photoaffinity labeling was performed at  $-196^{\circ}\text{C}$  with 100  $\mu\text{g}$  of PM protein, 0.5  $\mu\text{M}$  [ $^3\text{H}$ ]5N $_3$ -IAA, and 0.1 % Triton X-100.

Figure III.5. Intensity of photoaffinity labeling by [ $^3\text{H}$ ]5N $_3$ -IAA in the presence of unlabeled auxin or auxin analogs (1 mM): IAA (lanes 2 and 9), 1-naphthaleneacetic acid (lane 3), 1-naphthaleneacetic acid plus IAA (lane 4), 2-naphthaleneacetic acid (lane 6), 2,4-dichlorophenoxyacetic acid (lane 7), and tryptophan (lane 11). Controls (no added competitor) are shown in lanes 1 and 10. The fluorograph on the left (lanes 1-7) and the fluorograph on the right (lanes 8-11) are from two experiments performed on different days. Lanes 5 and 8 contain marker proteins; molecular weights are given between the fluorographs. Conditions were as for Figure III.4 at pH 6.5.

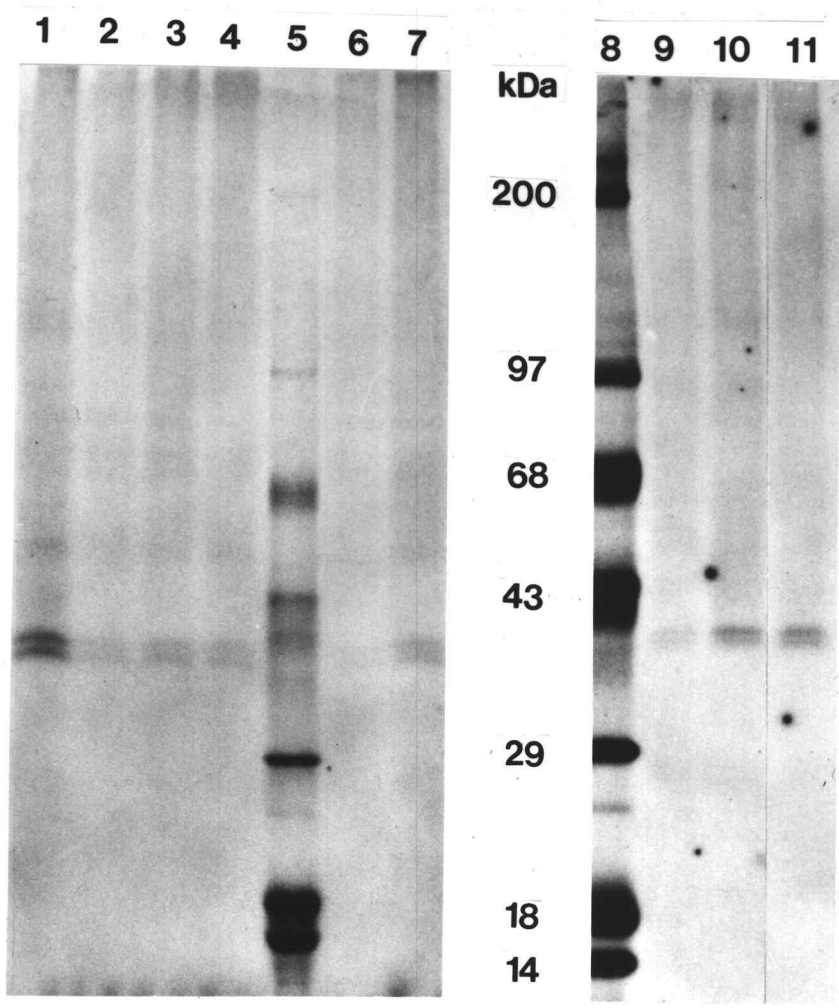


Figure III.5.



This result suggests that the auxin efflux carrier is not being labeled, since TIBA has been shown to specifically inhibit auxin association with this site in vivo (Rubery and Sheldake, 1974) and in vitro (Hertel et al., 1983; Jacobs and Hertel, 1978).

## Discussion

Plasma membrane vesicles from zucchini have been demonstrated to accumulate [ $^3\text{H}$ ]IAA *in vitro*. An integral component of this accumulation is believed to be an auxin-specific uptake symport (Hertel et al., 1983; Lomax et al., 1985). Here, we have shown that [ $^3\text{H}$ ]5N<sub>3</sub>-IAA is accumulated by these vesicles with competition characteristics similar to IAA. Thus, in theory, use of the azido-IAA should result in specific labeling of at least the auxin uptake carrier protein.

Our initial attempts to label auxin receptors in the PM with azido-IAA were carried out at 0-4°C and resulted in the labeling of a variety of polypeptides. Since the intensity of labeling was proportional to the abundance of PM proteins, it seems likely these conditions produce a high background of nonspecific labeling. Previous attempts to label auxin-binding proteins in microsomal membrane preparations from maize by photoaffinity labeling (Jones et al., 1984b) have also met with limited success as a result of non-specific labeling of membrane proteins. We have overcome this problem by lowering the temperature of the azido-IAA/membrane mixture to -196°C prior to photolysis. At this temperature, we observed the high specific-activity labeling of a low abundance polypeptide doublet of 40 kDa and 42 kDa with little labeling of other polypeptides. One possible reason that low

temperatures increase the specificity of photolabeling is that the residence time of the photogenerated nitrene at  $-196^{\circ}\text{C}$  is greater than the half life of this intermediate; thus diffusion of the reactive nitrene from the binding site to nonspecific sites would be greatly reduced. Another possibility may be the effect low temperatures have on the lipid phase present in these samples. Azido-IAA, being a lipophilic molecule, may partition less into the lipid phase below the phase transition temperature of the bilayer. This in turn would reduce nonspecific azido-IAA associations with the hydrophobic domains of membrane proteins.

The qualitative pattern of competition by auxin analogs for azido-IAA labeling (Figure III.5) is similar to that observed for accumulation of [ $^{14}\text{C}$ ]IAA into microsomal vesicles (Lomax, 1986) and [ $^3\text{H}$ ]IAA and [ $^3\text{H}$ ]5N<sub>3</sub>-IAA into PM vesicles (Table III.3). In particular, 2-naphthaleneacetic acid, which strongly competes for uptake and presumably the uptake carrier *in vitro*, prevents virtually all photoaffinity labeling, whereas 1-naphthaleneacetic acid, which competes less effectively for uptake, has a lesser effect. These data, along with the fact that the labeled polypeptides may be integral membrane proteins, suggest that the uptake carrier is being labeled. However, the putative PM auxin receptor site thought to be involved with proton secretion and thus with growth may have a similar specificity pattern and could not be distinguished from the uptake carrier by these experiments. The lack of interaction of TIBA with

azido-IAA labeling indicates that the efflux carrier responsible for the polar transport of IAA is not likely to be related to the 40 kDa and 42 kDa polypeptides labeled under these conditions. At this time we cannot fully explain why competition by auxin analogs requires higher than expected concentrations (1 mM) to substantially reduce labeling. However, this does not appear to be a unique problem since Ripp et al. (1988) encountered a similar difficulty using sucrose or other sugar analogs to compete with azido-sucrose when photolabeling a sucrose transport protein in soybean.

The fact that the two polypeptides are labeled indicates; either a) a multimeric protein, b) different processing states of the same protein e.g. preprotein, glycosylation, or proteolytic cleavage, or c) two different auxin-binding proteins. We do not know whether the azido-IAA labeled polypeptides reported here are related to auxin-binding proteins which have been isolated and solubilized by other means. In solubilization studies of naphthaleneacetic acid-binding proteins in maize, polypeptides of approximately 80 kDa (Cross and Briggs, 1978), 40 kDa (Venis, 1980), and approximately 20 kDa (Løebler and Klæmbt, 1985; Shimomura et al., 1986; Jones and Venis, 1988) have been isolated, some of which (Løebler and Klæmbt, 1985; Shimomura et al., 1986) appear to be of plasma membrane origin. It is possible that these polypeptides are related to one another as monomers of a multimeric protein, and the sizes might suggest that they are related to our polypeptides as well. However, this does not seem to be the case, since

the solubilized maize polypeptides exhibit different binding specificities and pH optima than those described here.

We found that preparative electrophoresis and electroelution (Figure III.3), yielded approximately 100 ng of each protein per 100 g fresh weight of zucchini hypocotyls. The yield was estimated by comparing the intensity of doublet staining to known quantities of control protein (data not shown). This is sufficient for the production of monoclonal antibodies and, perhaps, direct polypeptide microsequencing. Specific antibodies will allow both the immunological localization and characterization of the auxin-binding protein and the eventual reconstitution of the protein in an active form, which is a prerequisite for the ultimate identification of its role in auxin action.

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IV. THE *DIAGEOTROPICA* MUTANT OF TOMATO LACKS HIGH  
SPECIFIC ACTIVITY AUXIN BINDING SITES

### Abstract

Tomato plants homozygous for the *diageotropica* (*dgt*) mutation exhibit morphological and physiological abnormalities which suggest that they are unable to respond to the plant growth hormone auxin (indole-3-acetic acid). The photoaffinity auxin analog [<sup>3</sup>H]5N<sub>3</sub>-IAA specifically labels a polypeptide doublet of 40 kDa and 42 kDa in membrane preparations from stems of the parental variety, VFN8, but not from stems of plants containing the *dgt* mutation. In roots of the mutant plants, however, labeling is indistinguishable from that in VFN8. These data suggest that the two polypeptides are part of a physiologically important auxin receptor system, which is altered in a tissue-specific manner in the mutant.



## Introduction

The plant growth hormone auxin [indole-3-acetic acid (IAA)] appears to activate cellular responses, such as the promotion of cell elongation, by binding to specific receptor proteins. Evidence for auxin binding to both membrane-bound and soluble proteins has been reported (Rubery, 1981; Libbenga and Mennes, 1987). However, no direct experimental connection has been made between any plasma membrane auxin-binding protein and a known molecular or cellular response to auxin (Cross, 1985). Obtaining such evidence in conjunction with the isolation of the receptor would be important in elucidating the molecular mechanism of auxin action.

One way to investigate the physiological relevance of putative auxin receptors is through the use of mutant plants that are insensitive to auxin or which exhibit abnormalities likely to be influenced by IAA. The *diageotropica* (*dgt*) mutant of tomato (*Lycopersicon esculentum*, Mill.), is a recessive mutant of the parental variety, VFN8, and appears to have arisen spontaneously at a single locus. Tomato plants homozygous for the *dgt* mutation have diageotropic shoot growth, abnormal vascular tissue, altered leaf morphology, and no lateral root branching (Zobel, 1972b, 1973). Although the endogenous levels of auxin are the same in both *dgt* and VFN8 (Fujino et al., 1988), *dgt* mutants are insensitive to exogenously

applied auxin in ethylene production (Zobel, 1973; Fujino et al., 1988) and stem elongation (Kelley and Bradford, 1986). The morphological abnormalities exhibited by *dgt* plants, in addition to their inability to elongate in response to auxin, suggest that the *dgt* lesion is associated with a primary site of auxin perception or action such as an auxin-specific receptor.

To label and identify potential auxin receptors, we used a radioactively labeled photoaffinity auxin analog, [ $^3\text{H}$ ]5N<sub>3</sub>-IAA (azido-IAA). Azido-IAA is an active auxin in several different bioassays and its uptake and transport characteristics in stems are similar to those of auxin (Melhado et al., 1981; Jones et al., 1984a,b). Thus, one would expect azido-IAA to bind to auxin receptors with an affinity similar to that of auxin. In an earlier study on the binding of the azido-IAA to plasma membrane proteins from zucchini hypocotyls, vesicles were exposed to azido-IAA and photolyzed with ultraviolet light (300 nm) at -196°C (see Chapter III). Subsequent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography revealed that azido-IAA was associated at high specific activity with a polypeptide doublet of 40 kDa and 42 kDa. These polypeptides are of low abundance, as they cannot be visualized in silver-stained gels until they have been electroeluted and concentrated approximately tenfold. Competition experiments with various auxin analogs suggest that this doublet binds auxin specifically (see Chapter III). I report here on the application of the photoaffinity labeling

technique to test the hypothesis that the mutated *dgt* gene encodes a receptor protein which is present at abnormally low levels or has a greatly reduced ability to bind auxin.

## Materials and Methods

### Preparation of Membranes

Microsomes were prepared from stems or roots of 10-day-old etiolated seedlings of *dgt* and VFN8 tomato varieties. Material was homogenized with a Polytron for 15 s (level 8) in an equal amount (w/v) of ice-cold buffer I (10 mM tris-HCl, pH 7.5, and 0.25 M sucrose, 1 mM disodium-EDTA, 1 mM dithiothreitol, 0.1 mM MgSO<sub>4</sub>, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, and 1 µg/ml leupeptin). The homogenate was filtered through four layers of cheesecloth, and the remaining material reground with the same amount of buffer I. The combined filtrate was centrifuged at 4°C for 20 min at 3000x g and the pellet was discarded. The supernatant was centrifuged at 100,000x g for 30 min at 4°C, and the resulting microsomal pellet was suspended in buffer I, aliquoted, frozen in liquid N<sub>2</sub>, and stored at -80°C.

### Photoaffinity Labeling

For photoaffinity labeling (performed under red light), a quantity of microsomes equivalent to 100 µg of total protein (assayed according to Spector, 1978) was diluted to a final volume of 50 µl with buffer II (10 mM morpholinoethanesulfonic acid/bis[tris(hydroxymethyl)-methylamino]propane, pH 6.5, and 0.25 M sucrose), 0.5 µM

[<sup>3</sup>H]5N<sub>3</sub>-IAA (16 Ci/mmol) and Triton X-100 (final concentration 0.1%). Thirty sec after addition of microsomes, samples were transferred to glass cover slips, which were then placed on an aluminum block surrounded by liquid N<sub>2</sub>, and the samples were irradiated for 10 sec with 300 nm UV light. Irradiated samples were stored overnight in the dark at -20°C, washed by dilution into 1 ml buffer II, and centrifuged at 200,000x g for 5 min at 4°C. The pellets were resuspended in 20 µl of SDS loading buffer (62.5 mM tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.2 M dithiothreitol, and 0.25% bromphenol blue), and the proteins were separated by electrophoresis through 7.5% to 15% polyacrylamide gradient gels. After staining with Coomassie brilliant blue, gels were treated with a fluorographic enhancer, dried, and exposed to Kodak XAR-5 film.

## Results and Discussion

We have performed photoaffinity labeling experiments with microsomal membrane preparations from the hypocotyls (stems) or roots of 10-day-old etiolated VFN8 and *dgt* tomato seedlings (Figure IV.1). The silver-stained gel indicates that *dgt* and VFN8 microsomes do not display any obvious differences in their pattern of polypeptides, suggesting that *dgt* is indeed isogenic with VFN8 (Figure IV.1A). Both the roots and stems of VFN8 have a polypeptide doublet of 40 kDa and 42 kDa which is labeled to high specific activity with the azido-IAA (Figure IV.1B). The molecular weight of the tomato polypeptides agrees with those observed in zucchini. These labeled polypeptides were not detected in membranes from *dgt* stems after our usual fluorographic exposure time of 4 days, although the labeled doublet was present in *dgt* roots at an intensity equal to that in VFN8 roots (Figure IV.1B). Prolonged exposure (17 days) of these fluorographs produced a faint signal from *dgt* shoot preparations (Figure IV.1C). These data indicate that the 40 kDa and 42 kDa auxin-binding polypeptides are greatly diminished or have much reduced auxin binding capacity in the mutant stems. In either case, the alteration is developmentally regulated.

The presence of Triton X-100 (0.1%) during photolysis increases the intensity of polypeptide labeling by the azido-IAA in VFN8 stem

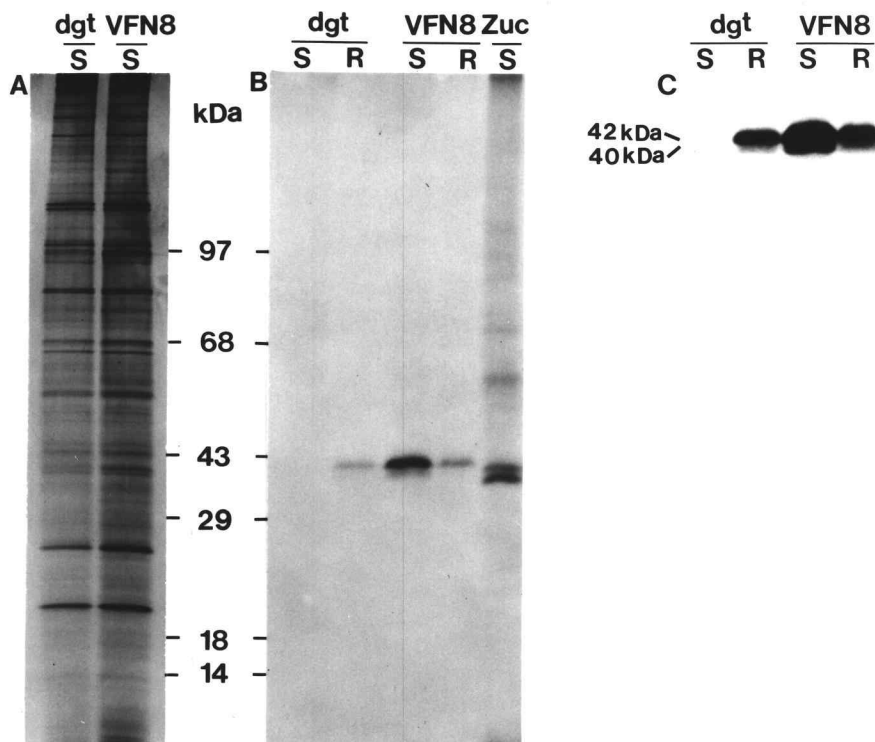


Figure IV.1. Differential high specific activity labeling of 40 kDa and 42 kDa polypeptides in microsomes of *dgt* and VFN8. (A) Silver-stained (Heukeshoven and Dernick, 1985) gel comparing unlabeled stem (S) microsomal polypeptides from *dgt* and VFN8. (B) Fluorograph (4 day exposure) showing polypeptide labeling in microsomes of stems (S) and roots (R) from *dgt* and VFN8. Labeled microsomal polypeptides of zucchini stems (zuc) are included for comparison. (C) Fluorograph resulting from a 17 day exposure of a portion of the gel shown in (B).

microsomes (Figure IV.2). As the data shown in Figure IV.1 were obtained in the presence of Triton X-100, it was important to demonstrate that the detergent does not preferentially remove the 40 kDa and 42 kDa doublet from *dgt* membrane preparations before gel electrophoresis. A selective removal of the proteins from the *dgt* membranes cannot explain the difference between the mutant and wild-type tissues in binding azido-IAA, since membranes exposed to azido-IAA in the absence of Triton X-100 also fail to yield bands which label with high specific activity in *dgt* stems (Figure IV.2). The fact that the labeled polypeptides in VFN8 remain with the sedimented membrane fraction after treatment with a relatively high concentration of detergent suggests that the polypeptides specifically labeled with azido-IAA may be intrinsic membrane proteins. We have obtained similar results with zucchini plasma membrane vesicles (see Chapter III).

The unusual phenotype of *dgt* plants and their insensitivity to auxin indicate that this mutant has an altered auxin receptor (Jackson, 1979; Bradford and Yang, 1980). Our results are consistent with this hypothesis. Microsomes from *dgt* shoots have greatly reduced amounts of the 40 kDa and 42 kDa polypeptides that efficiently bind azido-IAA. If this is an auxin receptor, the polypeptides should be (i) ubiquitous in plant tissues which respond to IAA, (ii) of low abundance, (iii) saturable with increasing concentrations of IAA, and (iv) competent with specific analogs that are also active auxins or



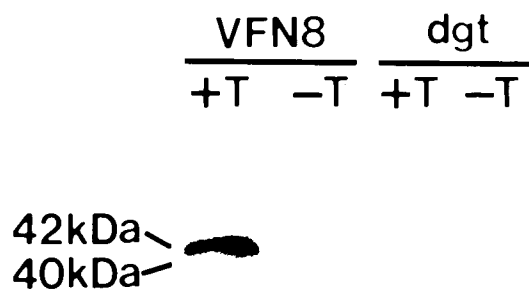


Figure IV.2. The effect of Triton X-100 on the azido-IAA photoaffinity labeling of polypeptides in VFN8 and *dgt* microsomes. Fluorograph (4 day exposure) showing labeled 40 kDa and 42 kDa polypeptides from VFN8 and *dgt* stems in the presence (+T) and absence (-T) of 0.1% Triton X-100. Photoaffinity labeling was as described (Figure IV.1).

specific antagonists. Azido-IAA labels a polypeptide doublet of this molecular weight range in various auxin-responsive tissues in many plant species such as *Arabidopsis*, maize and several conifers (Lomax and Hicks, 1992). Increasing concentrations of auxin protect the polypeptides from photoaffinity labeling in a corresponding manner (Figure IV.3, inset). Densitometer scans of the fluorographs (Figure IV.3) reveal an apparent half maximal saturation ( $K_{Da}$ ) at about  $10^{-5}$  M IAA. Similar  $K_{Da}$ 's for auxin binding have been previously reported (Rubery, 1981; Cross, 1985; Libbenga and Mennes, 1987). These results suggest that the photoaffinity labeling is saturable and, thus, probably reflects a specific interaction of the azido-IAA with a receptor molecule. Studies of zucchini microsomes and plasma membrane vesicles demonstrate that competition for the auxin binding exhibits a high degree of specificity for active auxins and auxin analogs (see Chapter III).

The *dgt* plants lack lateral roots, a developmental abnormality thought to be related to auxin (Wightman et al., 1980); yet membrane preparations from roots appear to have the 40 kDa and 42 kDa polypeptides with normal auxin-binding capacity. We have not resolved this apparent paradox. However, it has been reported that *dgt* roots produce a normal branching pattern when grafted to VFN8 shoots, whereas the reciprocal graft has no effect upon the morphology of mutant shoots or leaves (Zobel, 1974). Thus, we postulate that auxin-

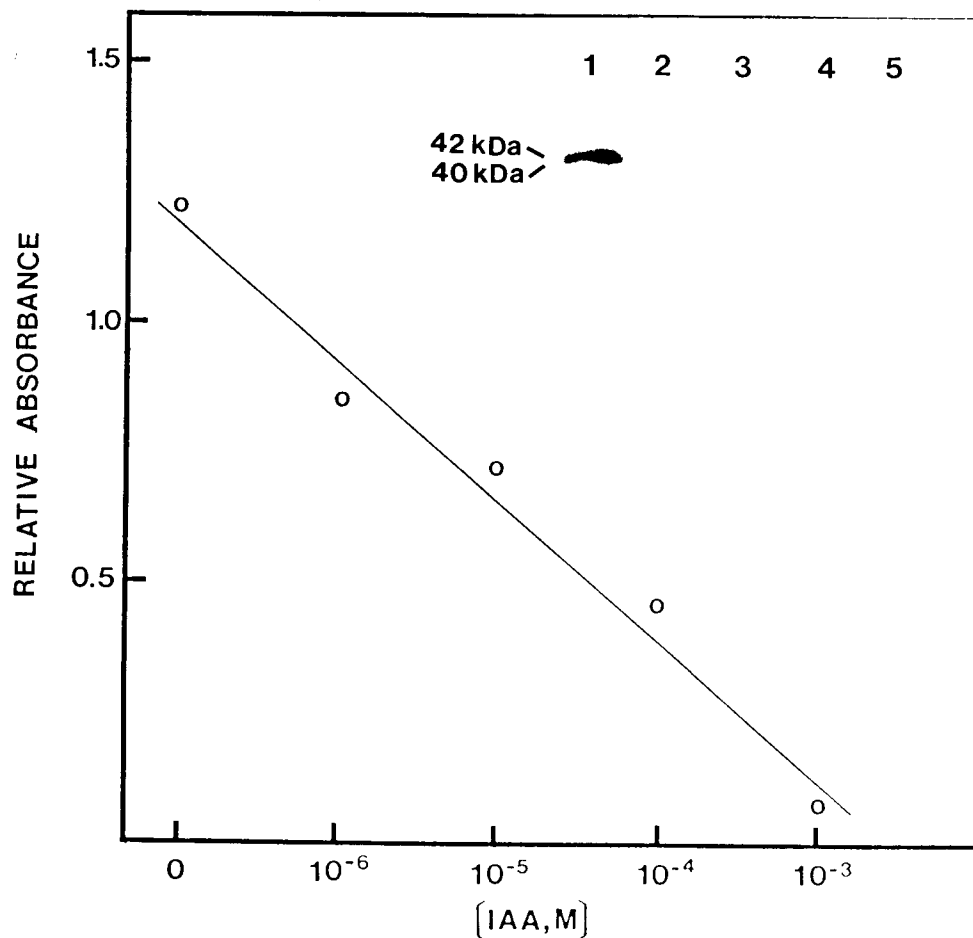


Figure IV.3. Reduction of 40 kDa and 42 kDa polypeptide labeling in microsomes of VFN8 stems by IAA . Inset: Fluorograph displaying polypeptide labeling in the presence of (1) zero, (2)  $10^{-6}$ M, (3)  $10^{-5}$ M, (4)  $10^{-4}$ M, or (5)  $10^{-3}$ M added IAA. Polypeptides of VFN8 stem microsomes were labeled as described in Figure IV.1. Line drawing shows results of a densitometer scan of the inset fluorograph. Relative absorbance (in arbitrary units) of the 42 kDa band was plotted as a function of the molar (M) concentration of added IAA.

sensitive stems produce a diffusible or transported factor that influences root branching. As *dgt* stems are auxin insensitive, they would not be expected to produce this factor. This hypothesis remains to be tested as does the question of whether *dgt* roots show some wild-type responses to exogenous IAA (for example, inhibition of elongation) as would be expected if they have a growth-specific auxin receptor.

The complex nature of the *dgt* phenotype is further demonstrated by more recent observations of variability in the relative intensity of photoaffinity labeling in membranes from mutant stems. By labeling a number of independently grown batches of seedlings, we found that the intensity of labeling in stems of VFN8 is quite consistent, whereas labeling in *dgt* can range from virtually undetectable (Figure IV.1) to an intensity nearly equal to that of VFN8 (data not shown). Extensive experimentation has demonstrated that the environmental conditions under which the mutant seedlings are grown is the likely source of variability. More specifically, the gaseous growth regulator, ethylene, may be implicated. Bradford and Yang (1980) reported that while *dgt* plants are incapable of producing ethylene in response to exogenous auxin, as can VFN8, they can produce this compound in response to stresses such as wounding. Preliminary results in our laboratory indicate that both mutant and wild-type seedlings grown in the presence of ethylene display a significantly reduced intensity of photoaffinity labeling relative to control seedlings grown in the presence of an ethylene specific absorbant (Hicks, Rayle and Lomax,

unpublished data). It may be hypothesized then that *dgt* stems are more sensitive than VFN8 stems to the inhibition of auxin binding by ethylene. More importantly, while the interactions of auxin and ethylene have been studied at the level of physiology, the 40 kDa and 42 kDa polypeptides may provide a unique opportunity to study the molecular interactions of these growth regulators.

There are several ways to explain the generally reduced levels of detectable azido-IAA labeling in *dgt* stems that are consistent with the finding that the azido-binding polypeptides are present in *dgt* roots. Perhaps the most straightforward explanation is that the structural gene (or genes) for the polypeptides is intact but because of an alteration of cis- or trans-acting regulatory factors it is expressed at a diminished level in the stem. It is also possible that there are separate genes expressed in root and stem with the latter being defective in *dgt*. Alternatively, the *dgt* lesion may affect post-translational processing, which prevents normal membrane insertion or folding of the polypeptides or reduces the affinity of the binding site for auxin in stem tissue.

It is believed that there are at least three plasma membrane-bound auxin receptors; an uptake symport (Rubery and Sheldrake, 1974; Hertel et al., 1983; Lomax et al., 1985; Benning, 1986; Lomax, 1986; Sabater and Rubery, 1987a,b), an asymmetrically distributed efflux carrier (Hertel and Leopold, 1963; Goldsmith, 1977; Jacobs and Gilbert,

1983), and a receptor associated with an outwardly directed proton pump thought to be involved in elongation growth (Rubery, 1981; Libbenga and Mennes, 1987). Present data do not allow us to distinguish between these possible receptor types. On the one hand, zucchini hypocotyl plasma membrane vesicles show a specificity for competition of azido-IAA labeling by auxin analogs which is similar to the specificity demonstrated for auxin uptake into both membrane vesicles and hypocotyl segments via the symport (see Chapter III). In addition, the ability of VFN8 shoots to normalize *dgt* roots could indicate that the *dgt* lesion alters either the uptake or efflux of auxin during cell-to-cell transport. However, other experiments indicate that the rate of polar auxin transport (for example, from shoot apex to base) is unimpaired in *dgt* stems as compared to that in VFN8 (Daniel et al., 1989). In addition, since *dgt* hypocotyl sections do not grow in response to externally applied auxin (Kelley and Bradford, 1986), the receptor responsible for auxin-stimulated growth may be affected by this lesion. The identification of the two polypeptides that appear to be affected by the *dgt* lesion may make it possible to dissect the mechanism of auxin action.

## Acknowledgements

This chapter appears under the same title by Glenn R. Hicks, David L. Rayle and Terri L. Lomax (Science 245, 52-54). I acknowledge Dr. Rayle and Dr. Lomax for conceptual contributions. I also acknowledge Dr. Rayle for introducing our laboratory to the *dgt* mutant and for assistance in growing plant materials. I thank K. Bradford for supplying the original stock of VFN8 and *dgt* seeds, A.M. Jones for generously providing the [<sup>3</sup>H]5N<sub>3</sub>-IAA, and R. Hopkins and I. Gelford for assistance with membrane preparation. Supported by National Science Foundation grant DCB-8718731 and National Aeronautics and Space Administration grants NAGW 1253 (to T.L.L.) and NAGW 230 (to D.L.R.).

V. CHARACTERIZATION OF AUXIN-BINDING PROTEINS FROM  
PLASMA MEMBRANES OF ZUCCHINI



## Abstract

We have previously reported the identification of several auxin-binding polypeptides in plasma membrane (PM) preparations from zucchini (*Cucurbita pepo*) (see Chapter III). These polypeptides have molecular weights of 40 kDa and 42 kDa and are specifically labeled with the photoaffinity auxin analog [<sup>3</sup>H]5N<sub>3</sub>-IAA (azido-IAA). Azido-IAA permits both the covalent and radioactive tagging of putative auxin receptors and has facilitated the further characterization and purification of the 40 kDa and 42 kDa polypeptides. Consistent with their possible role as a physiologically important auxin binding site, the azido-IAA labeled polypeptides are detected in auxin-responsive tissues such as hypocotyls and root but not in mature leaf tissue. The azido-IAA labeled polypeptides remain in the pelleted membrane fraction following high salt and detergent washes, which indicates a tight and integral association with the PM. Phase extraction with the nonionic detergent Triton X-114 results in partitioning of the azido-IAA labeled polypeptides into the aqueous (hydrophilic) phase. This paradoxical behavior has been hypothesized to be a characteristic of certain proteins that aggregate to form ionic channels through membranes. The results of gel filtration indicate that the auxin-binding proteins aggregate strongly and may associate to form a dimer *in vivo*. Two-dimensional electrophoresis of partially purified azido-IAA labeled protein demonstrates that the major isoforms of the 40 kDa

and 42 kDa polypeptides have isoelectric points (pIs) of 8.2 and 7.2, respectively, although other charge variants are apparent. Tryptic and chymotryptic digestion of the auxin-binding proteins strongly suggests that the 40 kDa and 42 kDa polypeptides are closely related or are modifications of the same polypeptide.

## Introduction

The plant hormone auxin (indole-3-acetic acid, IAA) is important in a variety of developmental processes such as growth and differentiation. At the cellular level, auxin may regulate these responses by interacting with specific membrane-localized and/or soluble receptors (for review see Rubery, 1981; Cross, 1985; Libbenga and Mennes, 1987; Napier and Venis, 1990). We are particularly interested in identifying and characterizing auxin binding sites at the plasma membrane (PM). These sites could be proteins involved in auxin transport such as the hypothesized uptake (Rubery and Sheldrake, 1974; Hertel et al., 1983; Lomax et al., 1985) or efflux carriers (Goldsmith, 1977; Jacobs and Gilbert, 1983) or a receptor involved in auxin stimulated growth (Rubery, 1981; Libbenga and Mennes, 1987).

The technique of photoaffinity labeling (for review see Bayley and Knowles, 1977; Chowdhry and Westheimer, 1979; Ruoho et al., 1984) by a tritiated azido-auxin analog ( $[^3\text{H}]5\text{N}_3\text{-IAA}$ , azido-IAA) has been used to localize possible sites of auxin transport (Jones, 1990). Azido-IAA has been demonstrated to have auxin activity in several bioassays (Melhado et al., 1981) and, therefore, has been utilized to identify and characterize putative receptors (Jones et al., 1984a, b; see Chapter III). We have found that ultraviolet irradiation (300 nm) of highly-purified

PM vesicles from zucchini (*Cucurbita pepo*) in the presence of azido-IAA results in the covalent, high specific activity labeling of a polypeptide doublet at 40 kDa and 42 kDa. Labeling of this doublet is particularly evident when photolysis is conducted at liquid nitrogen temperature (-196°C) (see Chapter III). The presence of auxins and auxin analogs during photolysis results in significantly reduced labeling indicating that labeling is specific for auxins. Polypeptides of similar molecular weights have been detected in membrane preparations from a variety of divergent plant species (Lomax and Hicks, 1992; see Chapter IV) which suggests that these polypeptides may be conserved.

Several additional auxin-binding proteins have been reported. The most extensively studied auxin-binding protein is the 22 kDa endoplasmic reticulum (ER) protein from maize which is known from auxin-binding studies as Site I (for review see Napier and Venis, 1990). Recently, a soluble 25 kDa polypeptide was found to be specifically labeled at -196°C by azido-IAA in *Hyoscyamus muticus* suspension cells (Mcdonald et al., 1991). A 60 kDa protein from maize PM was also labeled with azido-IAA though few details were reported (Palme et al., 1991). Prasad and Jones (1991) have utilized anti-idiotypic antibodies to identify a 65 kDa auxin-binding protein which is found in the nucleus and cytoplasm. In addition, the presumptive auxin efflux carrier has been photoaffinity labeled with an azido-naphthalphthlamic acid

(Palme et al., 1991). The relationship of the various auxin-binding proteins is uncertain and direct sequence comparison is not possible, since only the Site I ER protein from maize has been cloned and sequenced (Inohara et al., 1989; Tillmann et al., 1989; Hesse et al., 1989).

Here, we report on further characterization of the azido-IAA labeled 40 kDa and 42 kDa polypeptides. Labeling studies indicate that the polypeptides bind azido-IAA in all regions of etiolated hypocotyls and in roots and expanding leaves, but not in mature leaves. Salt and detergent treatment indicate that these proteins are tightly associated with the membrane, and gel filtration data suggest that the 40 kDa and 42 kDa polypeptides associate as a dimer *in vivo*. Two-dimensional electrophoresis and Triton X-114 phase partitioning were also performed. Finally, data from proteolytic digestion of partially purified material suggests that the 40 kDa and 42 kDa polypeptides are closely related.

## Materials and Methods

### Reagents, Plant Material, and Preparation of Membrane Vesicles

All reagents were from Sigma Chemical Co., St. Louis, MO, except where indicated. Hypocotyls and roots were excised from etiolated seedlings of zucchini (*Cucurbita pepo* L. cv. Dark Green, Ferry Morse Seed Co., Mountain View, CA) that were grown in moist vermiculite for 5 days at 28°C. Leaf tissue was excised from zucchini plants that were grown in a greenhouse for approximately 2 months. Microsome or purified plasma membrane (PM) vesicles were prepared from hypocotyl sections as previously described (see Chapters III, IV).

### Photoaffinity Labeling

Photoaffinity labeling at liquid nitrogen temperature (-196°C) in the presence or absence of 1 mM IAA as a competitor was conducted as previously described (see Chapters III, IV). For preparative SDS-PAGE, the reported procedures were modified as follows. A quantity of PM vesicles equivalent to 1 mg of protein, as assayed by Coomassie brilliant blue G-250 binding (Spector, 1978), was diluted to 500 µl with binding buffer (10 mM MES/1,3 bis[tris(hydroxymethyl)methylamino]propane, pH 6.0, 0.25 M sucrose) containing 0.1% Triton X-100 (Boehringer Mannheim Corp., Indianapolis, IN) in a UV transparent disposable cuvette (Fisher Scientific Co., Springfield, NJ). Azido-IAA (kindly provided by Dr. A. Jones, University of North Carolina) had a

specific activity of 16 Ci/mmol (1 Ci = 37 GBq) and was present at a final concentration of 0.5  $\mu$ M. The samples were cooled to -196°C and allowed to equilibrate for 5 min, followed by irradiation with 300 nm light for 15 sec. After irradiation, samples were stored overnight in the dark at -20°C. The PM vesicles were pelleted at 200,000x g for 5 min at 4°C (TLA 100.3 rotor, Beckman Instruments, Palo Alto, CA), suspended in 100  $\mu$ l of SDS-PAGE loading buffer and an amount of PM protein equivalent to three 1 mg labeling reactions (300  $\mu$ l) was fractionated by preparative electrophoresis through 1.5 mm 7.5% to 15% gradient SDS polyacrylamide gels (SDS-PAGE). Gels were stained with Coomassie brilliant blue R-250 (Hames, 1981) and fluorographed as described (see Chapter III).

The gel region corresponding to the labeled polypeptides was excised and electroeluted into 12.5 mM Tris/0.2 M glycine buffer containing 0.05% SDS according to Lomax (1978). The proteins electroeluted from 8 preparative gels (equivalent to 24 mg of total PM protein) were concentrated to approximately 100  $\mu$ l by filtration through a Centricon-10 concentrator (Amicon, Danvers, MA) and re-fractionated by two-dimensional gel electrophoresis according to O'Farrell (1975). Fifty  $\mu$ l of material was subjected to first dimension isoelectric focusing (IEF) in 3.5% ampholytes (Pharmolyte, Pharmacia Corp., Piscataway, NJ) for 6000 v/hrs. For the second dimension, the first dimension IEF products were electrophoresed through a 1.5 mm 7.5% to 15% SDS gradient acrylamide gel without a stacking gel. The

resulting gels were either silver-stained (Heukeshoven and Dernick, 1985) or Coomassie-stained and fluorographed.

For salt and detergent washes, a quantity of PM vesicles equivalent to 2 mg of protein was azido-IAA labeled in a final volume of 1 ml of binding buffer. Two-hundred  $\mu\text{g}$  aliquots (100  $\mu\text{l}$ ) were removed, to which 5 M NaCl or  $\beta$ -octylglucoside (Boehringer Mannheim) from a 10% aqueous stock was added to the final concentrations indicated. Samples were incubated on ice for 15 min before the PM vesicles were pelleted at 200,000x g for 10 min at 4°C. Proteins were precipitated from the pellet and supernatant fractions by the addition of acetone:ethanol (1:1) to 1.5 ml final volume. Following overnight precipitation at -20°C, protein was pelleted by centrifugation at 15,000x g in an Eppendorf microfuge for 15 min. Samples were washed with 200  $\mu\text{l}$  of 90% acetone, vacuum dried for 30 min, suspended in 20  $\mu\text{l}$  of SDS-PAGE loading buffer, and subjected to SDS-PAGE and fluorography.

#### Triton X-114 Extraction

A quantity of PM vesicles equivalent to 400  $\mu\text{g}$  of protein was azido-IAA labeled in a final volume of 200  $\mu\text{l}$  of binding buffer. Triton X-100 when present was at a final concentration of 0.1%. Triton X-114 extraction was essentially as described by Bordier (1981). All extraction steps were performed under a red light. Following overnight storage at -20°C, azido-IAA labeled PM vesicles were thawed and 1/10 volume of



10 X Tris salt buffer (100 mM Tris HCl, pH 7.6, 1.5M NaCl) was added. The solution was brought to a final concentration of 2% Triton X-114 (Boehringer Mannheim) from an aqueous 10% stock. Proteins were incubated on ice for 5 min, and the sample was centrifuged at 58,000x g for 15 min at 4°C yielding a detergent pellet. The supernatant (approximately 250 µl) was layered onto an equal volume of sucrose cushion buffer (0.25 M sucrose, 1 X Tris salt buffer, 150 mM NaCl, 0.06% Triton X-114), incubated at 30°C for 5 min to induce phase separation and centrifuged at 2500x g for 15 min at 25°C. The upper aqueous phase was removed and re-extracted by the addition of Triton X-114 to 1%. After incubation on ice for 5 min, the aqueous phase was relayered over the lower sucrose detergent phase, which was retained from the previous step. The sample was incubated at 30°C for 5 min and centrifuged as above. The resulting upper aqueous phase, lower detergent phase, and the detergent pellet were brought to 1.5 ml final volume by the addition of acetone:ethanol (1:1), allowed to precipitate overnight and centrifuged as described. The resulting pellet was analyzed by SDS-PAGE and fluorography.

### Gel Filtration

Four mg of azido-IAA labeled PM protein was extracted with Triton X-114 as described, but the aqueous phase was not acetone:ethanol precipitated. Rather, the aqueous phase containing the 40 kDa and 42 kDa polypeptides was concentrated to approximately 200 µl in a Centricon-10 concentrator, washed twice with 10 volumes of

10 mM Tris, pH7.6, 0.1% Triton X-100 and chromatographed through a 2 cm diameter column (Econocolumn, Biorad Laboratories, Richmond, CA) containing an approximately 65 ml bed volume of Sephacryl S-300 (Pharmacia). Prior to chromatography, the column was equilibrated at 4°C with the nondenaturing buffer indicated.

For gel filtration under denaturing conditions, a similar column was prepared, and extracted protein as well as the column packing were equilibrated at room temperature with the buffer indicated. Columns were pumped at a flow rate of 0.2 ml per minute and 1 ml fractions were collected of which 100 µl was assayed for radioactivity by scintillation counting. Fractions containing radiolabeled protein were pooled, acetone:ethanol precipitated and analyzed by SDS-PAGE and fluorography.

#### Tryptic and Chymotryptic Digestion

Radiolabeled 40 kDa and 42 kDa polypeptides were enzymatically digested in gel slices as described by Kehry and Dahlquist (1982). Gel slices containing labeled 40 kDa and 42 kDa polypeptides from preparative SDS-PAGE (1.5 mg PM protein applied) were suspended in 1 ml of 0.2 M N-ethylmorpholine acetate, pH7.4. Trypsin (1 µg) or chymotrypsin (1 µg) was added and digestion was allowed to proceed for 8 hours at 25°C. Peptide fragments which eluted from the gel slices were chromatographed through a 4.6 mm x 25 cm C18 column (Ultrasphere, Beckman) by reverse phase HPLC. HPLC solvents

(Pierce, Rockford, IL) and gradients are as indicated. Flow rates were 1 ml per minute, and the indicated fractions were collected and counted in scintillation cocktail (Ready Safe, Beckman).

## Results

### Tissue Specificity of [<sup>3</sup>H]5N<sub>3</sub>-IAA Labeling

To examine the tissue specificity of azido-IAA labeling, membrane vesicles were prepared from hypocotyls, roots and leaves of zucchini. Hypocotyl sections, 1 cm in length, were excised 1 to 4 cm below the apical hook, and PM vesicles were purified. The vesicles were then photolyzed in the presence of [<sup>3</sup>H]5N<sub>3</sub>-IAA, and the membrane proteins were fractionated by SDS-PAGE and visualized by Coomassie blue staining (Figure V.1a). Fluorography of this gel revealed the presence of labeling of the 40 kDa and 42 kDa auxin-binding polypeptides at an equal intensity in each of the hypocotyl sections examined (Figure V.1b). Photoaffinity labeling of membrane preparations from roots, expanding leaves (< 3 cm in width) and mature leaves (> 3 cm in width) of zucchini revealed radiolabeled polypeptides in roots (Figure V.1c, lanes 5,6,11,12) and expanding leaves (Figure V.1c, lanes 13,14) but not in mature leaves (Figure V.1c, lanes 15,16). In addition to radiolabeled proteins of approximately 40 kDa, expanding leaves possess several other azido-IAA labeled polypeptides.

The intensity of photoaffinity labeling observed in PM preparations from roots and hypocotyls was greater than that of the corresponding microsome preparation (Figure V.1c, compare lanes

Figure V.1. Photoaffinity labeling of membrane preparations from different tissues of zucchini. Membrane vesicles were prepared from regions of etiolated hypocotyls 1 cm (lanes 1), 2 cm (lanes 2), 3 cm (lanes 3), and 4 cm (lanes 4) below the apical hook and photolyzed in the presence of [ $^3\text{H}$ ]5N $_3$ -IAA. (a) Coomassie-stained SDS polyacrylamide gel of azido-IAA labeled PM preparation from hypocotyls (100  $\mu\text{g}$  protein per lane). (b) Matching fluorograph of gel is shown for comparison. Note reversal in the order of lanes. (c) Fluorograph of photoaffinity labeled microsome vesicles prepared from roots (lanes 5, 6), hypocotyls (lanes 7, 8), expanding leaves (lanes 13, 14), and mature leaves (lanes 15, 16). PM proteins that were prepared from hypocotyls (lanes 9, 10) and roots (lanes 11, 12) are included for comparison. Membrane vesicles were photolyzed in the presence (odd numbered lanes) or absence (even numbered lanes) of competitor IAA. Molecular weights of protein standards (m) are indicated.

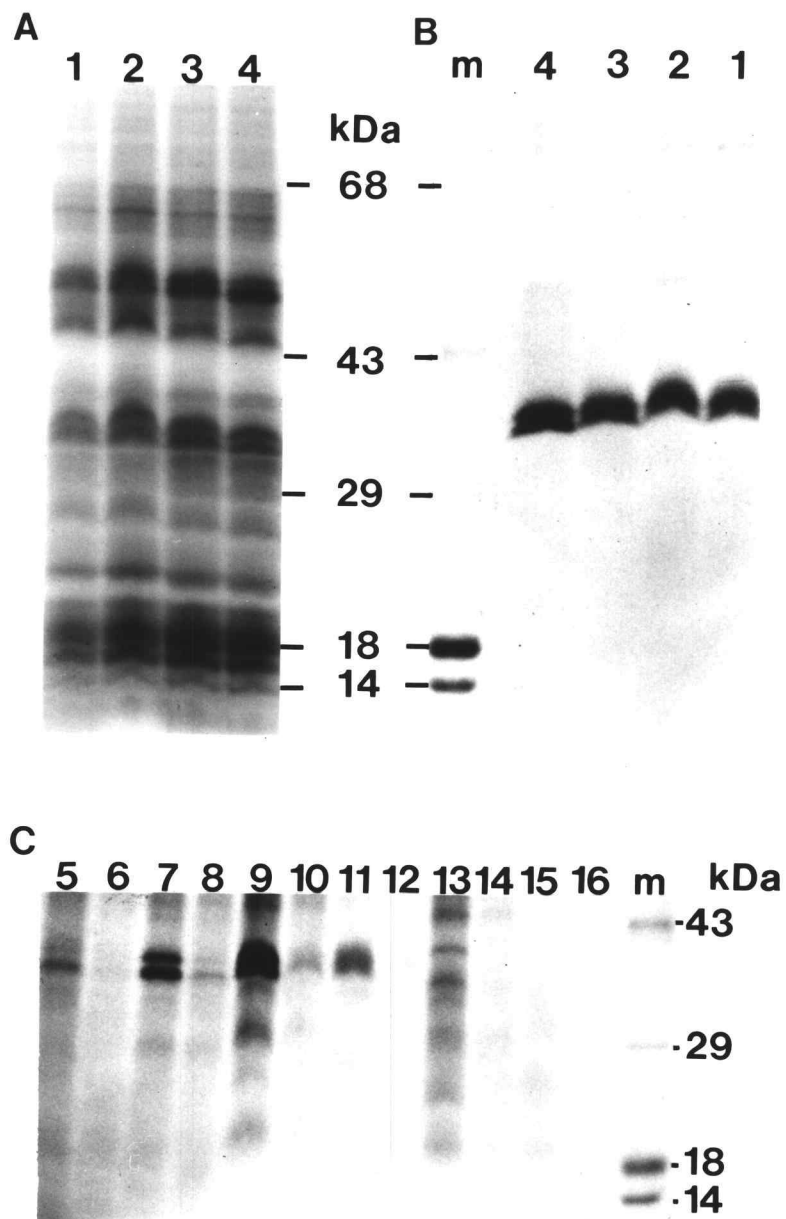


Figure V.1.

9-12 to lanes 5-8). This indicates that the 40 kDa and 42 kDa polypeptides are enriched in the PM. For each tissue examined, the presence of IAA (Figure V.1c, even numbered lanes) during photolysis significantly reduced azido-IAA labeling when compared to an absence of competitor (Figure V.1c, odd numbered lanes).

#### Membrane Association of the 40 kDa and 42 kDa Polypeptides

To determine the degree to which the 40 kDa and 42 kDa polypeptides are membrane associated, PM vesicles were photolyzed in the presence of azido-IAA, then incubated and pelleted in the presence of high salt or various concentrations of the nonionic detergent,  $\beta$ -octylglucoside. Both of the azido-IAA labeled polypeptides remained associated with the pelleted fraction (p) rather than the supernatant fraction (s) following a 15 min incubation in the absence (Figure V.2, lanes 1) or presence of 0.5 M NaCl (Figure V.2, lanes 2). The labeled proteins also remained associated with the pelleted fraction following incubation in  $\beta$ -octylglucoside at a concentration of 0.7%, which is equivalent to the critical micelle concentration (CMC; Figure V.2, lanes 3). When the CMC of the detergent was significantly exceeded (2%), the 40 kDa and 42 kDa polypeptides were found in the supernatant fraction (Figure V.2, lanes 4). These results indicate that the azido-IAA binding proteins are strongly associated with the PM.

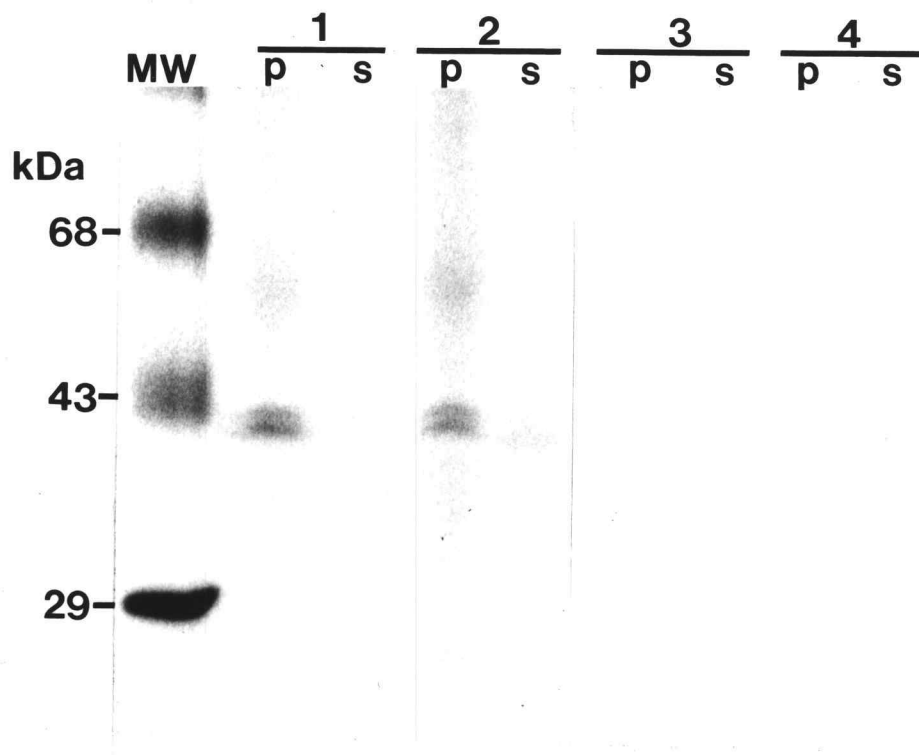


Figure V.2. Fluorograph of proteins that were azido-IAA labeled prior to treatment of PM vesicles with NaCl and detergent. PM vesicles were incubated in the absence (lanes 1) or presence of 0.5 M NaCl (lanes 2), 0.7%  $\beta$ -octylglucoside (lanes 3) or 2%  $\beta$ -octylglucoside (lanes 4). Membranes were pelleted, and the resulting pellet (p) and supernatant (s) fractions were analyzed by SDS-PAGE and exposed to film (XAR, Kodak) for approximately 5 days. Molecular weights of protein standards (MW) are indicated.



### Triton X-114 Extraction

Photoaffinity labeled PM protein was subjected to a temperature-induced phase separation in the nonionic detergent Triton X-114. Bordier et al. (1981) have reported that this method results in a convenient separation of hydrophilic (peripheral) and hydrophobic (integral) membrane proteins. PM vesicles from zucchini treated in this manner resulted in essentially distinct populations of proteins in the aqueous phase (Figure V.3A, lanes a), detergent phase (Figure V.3A, lanes d) and pellet (Figure V.3A, lanes p) as judged by SDS-PAGE and Coomassie blue staining. Upon fluorography, the 40 kDa and 42 kDa azido-IAA labeled polypeptides were found to partition quantitatively into the aqueous phase with the presumably hydrophilic and peripherally associated proteins (Figure V.3B, lanes a). This extraction procedure results in an approximately two-fold enrichment of the radiolabeled polypeptides as estimated from SDS-PAGE (Figure V.3A).

It was previously reported that UV irradiation in the presence of Triton X-100 increased the specific activity of photoaffinity labeling with azido-IAA (see Chapters III, IV). This observation is particularly evident upon fluorography of Triton X-114 extracted protein that was previously photoaffinity labeled (Figure V.3B, compare lanes + X-100 and - X-100).

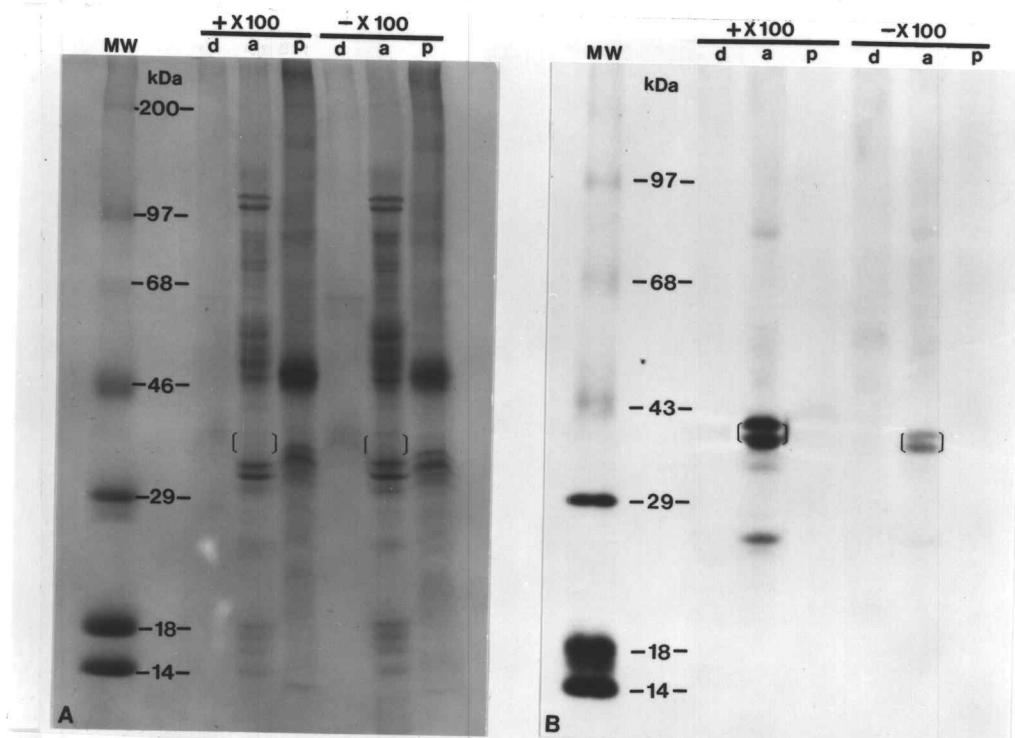


Figure V.3. Triton X-114 phase separation of photoaffinity labeled PM proteins. Following incubation with Triton X-114, samples were centrifuged to yield a detergent pellet (p). The supernatant was allowed to partition at 30°C and centrifuged to yield a detergent enriched phase (d) and detergent depleted aqueous phase (a). (A) Coomassie-stained SDS polyacrylamide gel of the resulting fractions. (B) Matching fluorograph is shown for comparison. Photolysis in the absence (lanes - X 100) or presence (lanes + X 100) of 0.1% Triton X-100 is also shown for comparison. Brackets indicate positions of the 40 kDa and 42 kDa polypeptides. Molecular weights of protein standards (MW) are as indicated.

### Gel Filtration of Triton X-114 Extracted Protein

The aqueous phase from Triton X-114 extraction was fractionated by Sephacryl S-300 gel filtration under non-denaturing conditions (Figure V.4A). Two radiolabeled peaks were detected: a broad peak of approximately 87 kDa and a sharper peak of 300 kDa or greater. Fractions from the radiolabeled peaks were pooled (Figure V.4A, indicated by horizontal bars), concentrated and analyzed by SDS-PAGE and fluorography (Figure V.4A, inset). Both peaks were found to contain azido-IAA labeled 40 kDa and 42 kDa polypeptides but no polypeptides of higher molecular weight. Fractions containing the 87 kDa peak were then rechromatographed through a similar gel filtration column under denaturing conditions. The resultant peak again eluted at approximately 87 kDa (Figure V.4B). As above, the radiolabeled peak was analyzed for 40 kDa and 42 kDa polypeptides by SDS-PAGE and fluorography (Figure V.4B, inset). None of the denaturing conditions employed caused the 87 kDa peak, which is presumably a dimer, to elute at approximately 40 kDa.

In a similar experiment, the peak at greater than 300 kDa did not yield peaks at either 87 kDa or 40 kDa under denaturing conditions. Upon SDS-PAGE and fluorography of this greater than 300 kDa peak, however, only radiolabeled polypeptides at 40 kDa and 42 kDa were observed (data not shown).

Figure V.4. S-300 Sephacryl gel filtration. (A) Azido-IAA labeled PM proteins were extracted with Triton X-114, and the aqueous phase was chromatographed in 10 mM Tris HCl, pH 7.6, 100 mM NaCl, 0.1% Triton X-100. (B) Fractions containing protein from the 87 kDa peak were pooled and chromatographed in 10 mM Tris HCl, pH 7.6, 100 mM NaCl, 1% SDS. One hundred  $\mu$ l of each 1 ml fraction was analyzed for radioactivity by scintillation counting, and selected fractions containing radioactivity (indicated by bars 1 to 5) were analyzed by SDS-PAGE and fluorography (see insets). The column was calibrated with the molecular weight standards blue dextran (void, v), bovine serum albumin (68 kD), carbonic anhydrase (30 kD), and cytochrome c (12 kD).

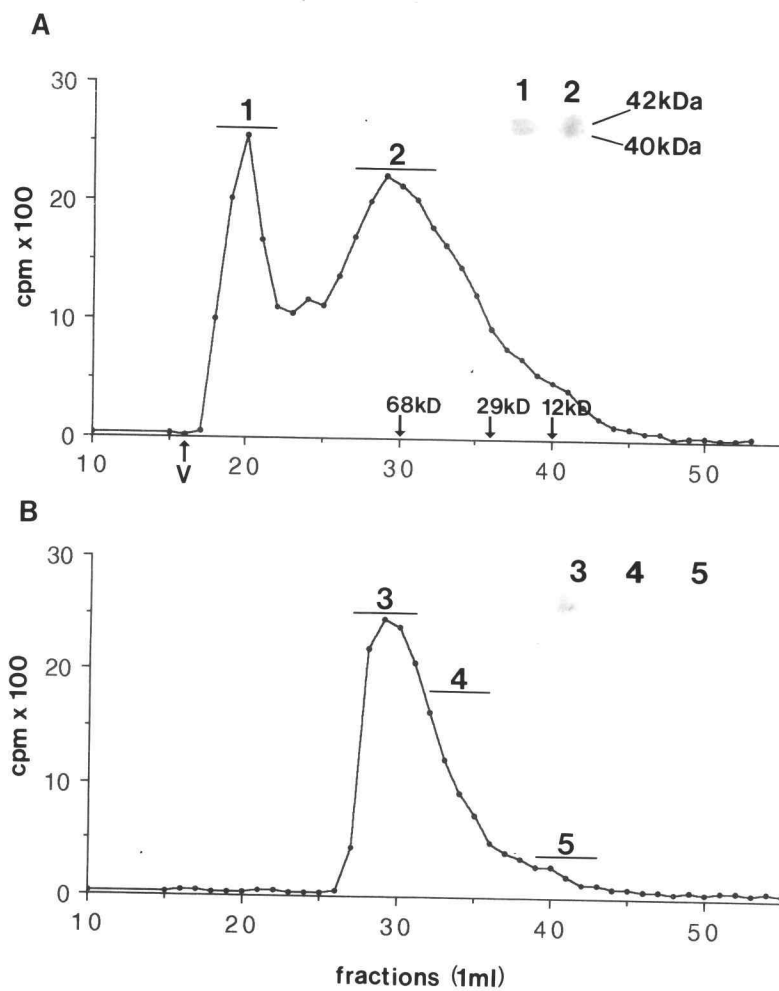


Figure V.4.

### Two-dimensional Electrophoresis of the Azido-IAA Labeled Polypeptides

Two-dimensional gel analysis and fluorography of azido-IAA labeled proteins enriched by preparative SDS-PAGE indicates that the 40 kDa and 42 kDa polypeptides correspond to proteins that are detectable by silver staining and have isoelectric points (pIs) of approximately 8.2 and 7.2, respectively (Figure V.5A). Aliquots of the same SDS-PAGE-enriched protein were electrophoresed through identical two-dimensional gels and one was subjected to fluorography whereas the other was silver stained. Alignment of the fluorograph with the stained gel indicates that the stained spots correspond to radiolabeled polypeptides (Figure V.5B, circled spots). In addition to the radiolabeled pI 8.2 and 7.2 spots, numerous fluorographic spots are detected which appear to represent more acidic charge forms of the presumed parent pI 8.2 and 7.2 spots (Figure V.5B). These additional spots were not detectable by silver staining in this experiment (Figure V.5A) but are detectable in more heavily loaded gels (data not shown).

### Enzymatic Digestion of 40 kDa and 42 kDa Polypeptides

Preparative gel slices containing either the photoaffinity labeled 40 kDa or 42 kDa polypeptide were incubated with the proteases trypsin or chymotrypsin. The resulting digestion products were analyzed for the presence of radiolabeled peptides, which are fragments of the azido-IAA labeled 40 kDa and 42 kDa polypeptides. Digestion of the SDS-PAGE enriched 42 kDa sample with trypsin followed by reverse phase HPLC analysis resulted in two radiolabeled peaks which eluted at

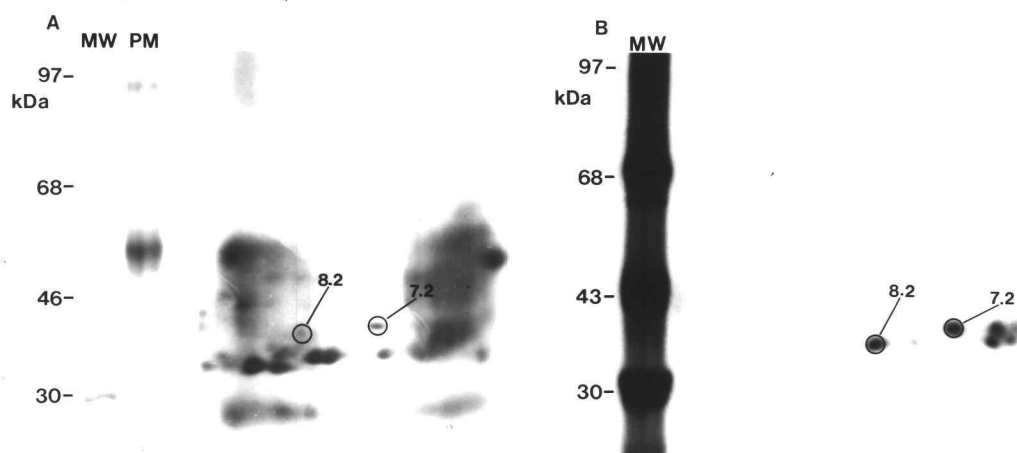


Figure V.5. Two-dimensional electrophoresis of azido-IAA labeled polypeptides. Proteins were enriched by preparative SDS-PAGE, and the region between approximately 35 kDa and 50 kDa was excised, electroeluted, concentrated and applied to first dimension IEF in pH 3 to 10 ampholytes. The second dimension was SDS-PAGE. A silver-stained gel (A) and a fluorograph produced from an identical gel (B) are shown for comparison. Circles indicate positions of the pI 8.2 and 7.2 proteins which are visible in the stained gel and the corresponding spots on the fluorograph. Molecular weight standards (MW) and unlabeled PM proteins (PM) are indicated.

approximately 32% and 33% acetonitrile (ACN) (Figure V.6A). When treated in a similar manner, samples enriched for the 40 kDa polypeptide yielded two radiolabeled peaks having retention times essentially identical to those of the 42 kDa sample (Figure V.5A). Control samples to which no enzymes were added yielded no radiolabeled peaks with similar retention times (data not shown).

Samples of the 40 kDa and 42 kDa polypeptides were also digested with chymotrypsin. As with trypsin, chymotryptic digestion resulted in the detection of 2 radiolabeled peaks for both the 40 kDa and 42 kDa samples (Figure V.5B). Furthermore, the 42 kDa peptide peaks eluted at essentially the same ACN concentrations (36% and 37% ACN) as the 40 kDa peptide peaks (Figure V.5B).

It should be noted that the percent ACN at which radiolabeled peptide elution occurred is different for the tryptic and chymotryptic digestion products. This suggests that the respective peptide fragments produced are of different lengths or amino acid composition. Overall, these results indicate that the 40 kDa and 42 kDa polypeptides are closely related proteins or may be different modification states of the same polypeptide.



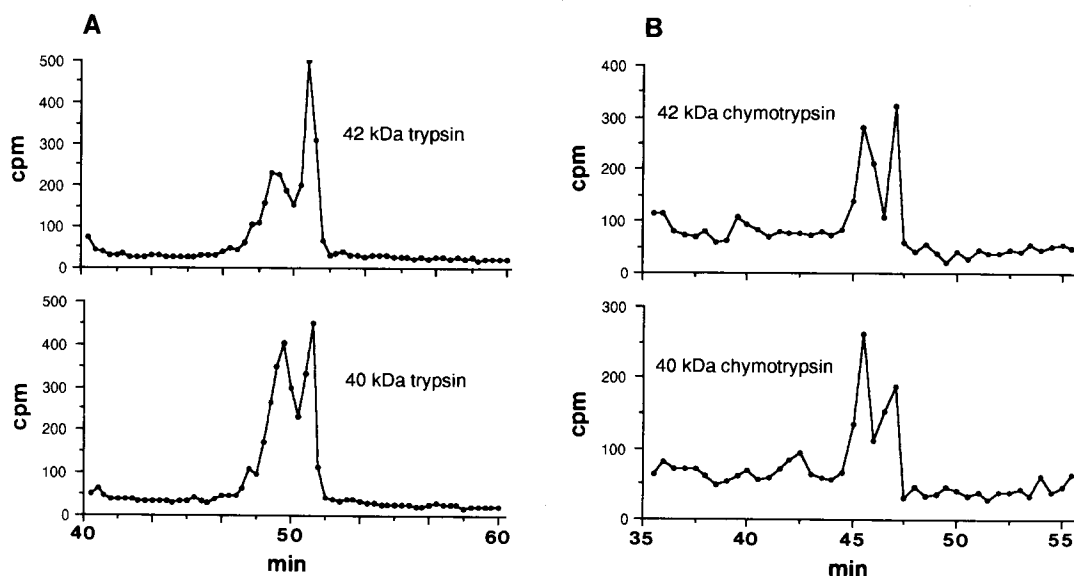


Figure V.6. Protease digestion and HPLC analysis of labeled polypeptides. Gel slices containing radiolabeled 40 kDa or 42 kDa polypeptides (approximately 3000 cpm) were digested with trypsin or chymotrypsin. Peptides were analyzed by C18 reverse phase HPLC using an ion-pairing gradient. Solvents were A: H<sub>2</sub>O + 0.1% TFA (trifluoroacetic acid) + 0.1% TEA (triethylamine) and B: ACN + 0.1% TFA + 0.1% TEA. For tryptic peptides (A), the gradient was 20% to 40% B over a period of 60 min, beginning at 13 min, and 0.3 min fractions were collected. For chymotryptic peptides (B), the gradient was 0% to 40% B from 3 to 50 min, and 0.5 min fractions were collected. Each fraction received 3 ml of scintillation fluid and was counted.

## Discussion

In this study (Figure V.1) and others (see Chapter IV), the presence of azido-IAA binding polypeptides in auxin responsive tissues such as hypocotyl and root (Taiz and Zeiger, 1991) but not in mature leaves suggests that the 40 kDa and 42 kDa polypeptides constitute a physiologically relevant auxin binding site. While the functional identity of the azido-IAA binding proteins remains uncertain, available evidence indicates that they are either an auxin specific uptake symport or a receptor associated with an outwardly directed pump which is thought to be involved in elongation growth (for review see Lomax and Hicks, 1992). The fact that an equal intensity of labeling is observed in membrane preparations derived from many regions of the hypocotyl (Figure V.1) and is not restricted to the zone of auxin induced cell elongation (upper 2 cm) suggests that the uptake symport is being labeled. However, in the regulation of growth, it is possible that the local concentration of auxin is more significant than the affinity of receptors for auxin. Photoaffinity labeling is observed in expanding leaf tissue (Figure V.1). The identity of the labeled proteins is unclear, however, since only several are at approximately 40 kDa. The fact that the azido-IAA binding polypeptides are enriched in PM vesicles from hypocotyls and roots provides evidence that these proteins are localized in the PM (Figure V.1).

The azido-IAA labeled 40 kDa and 42 kDa polypeptides are not solubilized from PM vesicles by relatively stringent washes with NaCl or detergent (Figure V.2). In addition, washes with Na<sub>2</sub>CO<sub>3</sub> fail to solubilize the proteins (data not shown). These results indicate that the 40 kDa and 42 kDa polypeptides are integral PM proteins. The behavior of the azido-IAA labeled proteins during the Triton X-114 partitioning (Figure V.3) appears to contradict this conclusion, since several reports indicate that hydrophobic membrane proteins, a term often used synonymously with integral membrane protein, are extracted almost exclusively into the detergent rich phase and pellet (Bordier, 1981; Pryde and Phillips, 1986). However, subsequent reports have demonstrated that certain integral membrane proteins such the acetylcholine receptor or the major anion transporter of erythrocytes, known as band three, partition into the aqueous phase (Maher and Singer, 1985; Pryde, 1986). In the case of the acetylcholine receptor, Maher and Singer (1985) hypothesize that the receptor possesses an unusual surface topography which leads to inefficient packing into detergent micelles. Therefore, the protein, though integral, is excluded from the detergent phase and pellet. It is also suggested that this behavior could be a general phenomenon of polypeptides that associate to form multisubunit hydrophilic charged channels through membranes. By analogy, it is possible that the 40 kDa and 42 kDa polypeptides might also have a channel function. Amino acid sequence would provide useful information regarding both the hydrophobicity and the potential cellular function of these proteins.

The results from gel filtration suggest that the azido-IAA binding proteins associate to form a dimer *in vivo* (Figure V.4). The tight association of the polypeptides is demonstrated by the fact that they eluted at the higher molecular weight (87 kDa) even under denaturing conditions during gel filtration. It could be argued that under native conditions the 87 kDa peak is a result of protein solubilized into Triton X-100 detergent micelles (molecular weight 90 kDa; Ausubel et al., 1989). However, gel filtration with detergents such as CHAPS and  $\beta$ -octylglucoside which form much smaller micelles had no effect on the apparent molecular weight (data not shown). Under denaturing conditions, increasing the NaCl concentration to 0.5 M or the addition of DTT (dithiothreitol) to 0.2% also had no effect on elution of the 87 kDa peak (data not shown). Another possibility is that the azido-IAA binding proteins may possess an asymmetric shape. For example, transcription factor TFIIB from yeast has been hypothesized to be ellipsoidal in shape which results in an apparent molecular weight that is much greater by gel filtration (248 kDa) than that observed by SDS-PAGE (60 kDa; Klekamp and Weil, 1987).

The peak at greater than 300 kDa is apparently a high molecular weight aggregate of the 40 kDa and 42 kDa proteins. It is interesting to note that Cross and Briggs (1978) reported a large aggregate peak (200 kDa) of auxin binding upon gel filtration of the Site I auxin-binding protein at low ionic strength. Upon increasing the NaCl concentration

to 100 mM, however, this peak was reduced in size. As discussed, increasing the salt concentration had no effect on either the 87 kDa or the greater than 300 kDa azido-IAA binding protein aggregates.

The 40 kDa and 42 kDa polypeptides (pIs 8.2 and 7.2, respectively, Figure V.5) are more basic than the only other auxin-binding protein for which the pI has been reported. This protein, the 22 kDa auxin-binding protein from maize ER, binds azido-IAA and has a pI of 5.0 to 5.3 (Jones and Venis, 1989). Thus, it is unlikely that the PM 40 kDa and 42 kDa azido-IAA binding proteins are aggregates or modifications of the 22 kDa protein. At this time, the nature of the various charge and molecular weight isoforms of the labeled proteins remain unclear, although there are obviously many possible explanations including glycosylation or phosphorylation. We have labored to exclude artifacts associated with two-dimensional gels such as carbamylation (Dunbar, 1987) by the use of deionized reagents and careful monitoring of the urea temperature. It is also possible that the azido-IAA may effect the pIs if it reacts with more than one site on the auxin-binding protein. Under these conditions, the charged carboxyl moiety of the azido-IAA which does not participate in nitrene formation may effect the net charge of the proteins.

Tryptic and chymotryptic digestion of the azido-IAA binding proteins provides useful information concerning the relationship of the 40 kDa to the 42 kDa polypeptide. The fact that two radiolabeled peaks

are obtained upon digestion and HPLC analysis of the labeled proteins (Figure V.6) suggests that there may be more than one site of azido-IAA binding per polypeptide. Alternatively, there may be only a single binding site, and one peak could represent an incomplete digestion product. This latter possibility seems unlikely, since longer digestion times do not yield fewer peptide fragments (data not shown). Finally, the peaks may result from minor differences in the amino acid compositions of the various charge isoforms. The resolution of C18 reverse phase HPLC and ion pairing gradients is sufficient to detect a single amino acid charge difference in a peptide of 20 to 30 residues, which is the probable length of the tryptic and chymotryptic fragments produced.

Regardless of the cause of the multiple peaks, the enzymatic digestions indicate that that 40 kDa and 42 kDa polypeptides are closely related proteins, at least in regions proximal to the site of azido-IAA labeling. It is possible that the 42 kDa polypeptide is a glycosylated form of the protein. For example, the 22 kDa auxin-binding protein from maize ER, which has a single N-linked glycosylation site, was found to have an apparent molecular weight of approximately 19 kDa upon digestion with endoglycosidase-H and analysis by SDS-PAGE (Inohara, 1989). It should be noted, however, that to date endoglycosidase-H and glycosidase-F digestions of partially purified preparations of the azido-IAA binding proteins have not yielded a detectable shift in molecular weight (data not shown). Alternatively,

the 42 kDa polypeptide may possess additional amino acids not found in the 40 kDa protein, which would account for the greater molecular weight. This question may be resolved when amino acid sequence is available.

In addition to pI and glycosylation state, other comparisons between the 40 kDa and 42 kDa azido-IAA binding proteins and the 22 kDa maize ER protein are possible (Table V.1). Both proteins appear to form dimers under native conditions, are protein doublets by SDS-PAGE, and are found in both dicot and monocot species, but this is the extent of their similarity. Evidence for the localization of the maize protein to the ER is convincing (Jones, 1990; Palme et al., 1991), whereas the azido-IAA binding proteins are enriched in the PM. The entire amino acid sequence of the ER protein reveals no obvious hydrophobic sequences except for a signal sequence (Hesse et al., 1989; Inohara et al., 1989; Tillman et al., 1989), while the PM proteins are integral membrane proteins which suggests considerable hydrophobic amino acid content. The abundance of the 40 kDa and 42 kDa proteins (estimated to be 0.0001% of total protein; Hicks and Lomax, unpublished) is also less than that of the 22 kDa protein (0.01% of total protein; Shimomura et al., 1988). The proteins also display differences in their relative affinities for auxins such as IAA and naphthaleneacetic acid (Løebler and Klæmbt, 1985a; Shimomura et al., 1986; see Chapter III).

Table V.1. Comparison of auxin-binding proteins

	<u>40-42 kDa PM</u>	<u>22 kDa ER</u>
Molecular Weight	40-42 kDa (1) (87 kDa dimer)	20-22 kDa (2) (40-45 kDa dimer) (3)
Intracellular Localization	Plasma Membrane	ER Lumen (4,5)
Hydrophobicity	Integral Membrane Protein	Hydrophilic (2)
pI	7.2-8.2	5.0-5.3 (6)
Abundance	Low (0.001%) (7)	Higher (0.01%) (8)
Relative Affinity	IAA High, 1-NAA Low (1)	1-NAA High, IAA Low (9)
Species Distribution	Douglas Fir, Cedar, Pine, Oat, Maize, Zucchini, Tomato, <i>Arabidopsis</i> (10)	Maize, <i>Arabidopsis</i> , Strawberry, Spinach (5,11)

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1. see Chapter III

2. Hesse et al., 1989; Inohara et al., 1989; Tillmann et al., 1989

3. Shimomura et al., 1986

4. Jones et al., 1990

5. Palme et al., 1991

6. Jones and Venis, 1989

7. Hicks and Lomax, unpublished

8. Shimomura et al., 1988

9. Løebler and Klæmbt, 1985; Shimomura et al., 1986

10. Lomax and Hicks, 1992

11. Macdonald et al., 1991

1-NAA, 1-naphthaleneacetic acid



In terms of their functional roles in plant growth and development, there is evidence that the 22 kDa ER protein is involved in auxin-stimulated growth, although it is not yet convincing (for reviews see Jones, 1990; Cross, 1991). There is also evidence that the 40 kDa and 42 kDa proteins are involved in growth (see Chapter IV), while other evidence suggests a role in auxin transport (Lomax and Hicks, 1992). Research is in progress to further define the function of this site, and soon it may be possible to construct a coherent model of auxin action at the molecular level.

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## VI. SUMMARY AND CONCLUSIONS

This chapter will summarize the major observations reported in this thesis. It will also discuss the fundamental question of the functional role of the azido-IAA binding proteins and indicate future research directions toward answering this question.

The techniques of photoaffinity labeling with [<sup>3</sup>H]5N3-IAA (azido-IAA), as originally reported by Jones et al. (1984b), was modified by the use of liquid nitrogen temperature (-196°C) and a short period of UV irradiation (10 to 15 seconds). This resulted in the photoaffinity labeling of two polypeptides of 40 kDa and 42 kDa in PM vesicles of zucchini with a specificity similar to that expected of an auxin-specific receptor or transporter. Of the three hypothesized PM sites of auxin association, (uptake and efflux carriers and a receptor associated with growth), the efflux site is not likely to be involved because the specific efflux inhibitor 2,3,5-triiodobenzoic acid (TIBA) did not compete with azido-IAA for photoaffinity labeling.

Further characterization of the labeled polypeptides has revealed that the proteins are integral polypeptides that are enriched in the PM. Even though they are apparently hydrophobic, the azido-IAA labeled proteins partition into the aqueous phase following Triton X-114 partitioning. This behavior may be characteristic of channel-forming

proteins (Maher and Singer, 1985). The polypeptides appear to form a dimer of 87 kDa under native conditions and their isoelectric points have been determined by two-dimensional gel electrophoresis. These gels also indicate the presence of charge isoforms which likely indicate post-translational modifications such as multiple phosphorylations. Peptide mapping of radiolabeled fragments reveals that the 40 kDa and 42 kDa proteins are closely related and represent modifications of a single protein sequence.

The physiological significance of the azido-IAA binding proteins is indicated by their presence in auxin-responsive tissues such as hypocotyl and root from zucchini and a diverse variety of other plant species (Lomax and Hicks, 1992). In addition, the labeling of these proteins is generally reduced in the auxin-resistant tomato mutant *diageotropic* (*dgt*).

The most fundamental question to be addressed is the function of the 40 kDa and 42 kDa azido-IAA binding proteins. There is evidence which indicates that the labeled proteins are a receptor involved in growth. Photoaffinity labeling of the polypeptides is, with the exception noted, reduced in membrane vesicles from hypocotyls of the tomato mutant, *dgt*. This mutant has been suggested to be defective in its ability to perceive auxin with respect to hypocotyl elongation and ethylene production (Kelly and Bradford, 1980; Daniel and Rayle, 1989) even though the endogenous amounts of IAA are the same in shoots of

*dgt* and its parent, VFN8 (Fujino et al., 1988). As previously noted, the mechanism of ethylene production is unaffected by the mutation to the extent that anaerobiosis and wounding stimulate ethylene production in *dgt*. In addition, *dgt* hypocotyls elongate in response to fusicoccin, a fungal toxin which stimulates H<sup>+</sup> secretion by a mechanism not involving auxin-perception (Marre, 1979), and acidic mediums (Daniel and Rayle, 1989). This indicates that *dgt* hypocotyls are competent for growth, and the lesion affects IAA perception rather than growth or ethylene synthesis mechanisms. Furthermore, the polar transport of IAA is similar in *dgt* and VFN8 which would appear to preclude the involvement of the azido-IAA binding protein in this process (Daniel and Rayle, 1989).

There are several types of evidence which indicate that the 40 kDa and 42 kDa polypeptides are involved in auxin transport. When the relative abilities of auxins and auxin antagonists to inhibit the uptake of [<sup>14</sup>C]IAA, [<sup>3</sup>H]IAA and [<sup>3</sup>H]5N<sub>3</sub>-IAA in vitro is compared to their relative efficiencies in blocking photoaffinity labeling, a similar order is observed. It should be noted that this is a qualitative argument, since the concentrations of analogs necessary for competition for labeling are greater than those for inhibition of uptake. The reason for this behavior is unclear, however, it has been observed with other photoaffinity probes (Ripp et al., 1988). This phenomenon may be due to the covalent attachment of the azido-IAA to a site which

is proximal to a site normally occupied by IAA. Thus, inhibition would not be competitive in nature.

Studies of the spatial distribution of the azido-IAA binding proteins in hypocotyls of zucchini also indicate that a transport component has been identified. Labeling is observed at equal intensities throughout the hypocotyl and does not coincide with the zone of auxin-induced cell elongation, whereas the transport of auxin occurs along the entire length of the hypocotyl. Partitioning in the nonionic detergent Triton X-114 provides additional evidence of transporter function. The 40 kDa and 42 kDa azido-IAA labeled proteins display the anomalous behavior of partitioning into the aqueous (hydrophilic) phase even though they are apparently hydrophobic, integral membrane proteins. Other workers have reported this phenomenon and have suggested that it may be a general phenomenon of proteins that associate to form hydrophilic channels through membranes (Maher and Singer, 1985). The acetylcholine receptor and the major anion transporter of erythrocytes are examples (Pryde, 1986), and by analogy, the azido-IAA binding proteins may have a channel function.

The functional identification of these proteins will be a difficult process. This is indicated by the fact that the cDNAs to the 22 kDa auxin-binding protein of the endoplasmic reticulum (ER) have been available since 1989 (Hesse et al., 1989; Inohara et al., 1989; Tillmann et

al., 1989), yet questions still remain concerning their functional identity (Jones, 1990a). Nevertheless, cDNA cloning of the azido-IAA binding proteins will be a valuable step forward. At the very least, DNA sequence will permit a comparison to the ER protein for homology and may provide clues as to function by a comparison with known channel and receptor proteins. A more direct approach to functional identification would involve the specific use of antibodies in the tobacco protoplast system of Barbier-Brygoo et al. (1989). If involved in growth, antibodies to the 40 kDa and 42 kDa polypeptides would block hyperpolarization, as do heterologous antibodies to the 22 kDa auxin-binding protein of the ER. Normalization of *dgt* mutant plants by the introduction of expressed cDNAs or perhaps, more reasonably, inducing the *dgt* phenotype in VFN8 plants by the introduction of antisense gene constructs would provide strong evidence for an involvement in auxin-induced growth. To test transport function, it may be possible to express cDNAs in bacterial or yeast systems and to assay for the uptake of radiolabeled auxins. Alternatively, it should be feasible to express cDNAs or mRNAs synthesized in vitro in *Xenopus* oocytes and again assay for uptake.

To propose a specific model concerning the cellular role of the 40 kDa and 42 kDa polypeptides would be highly speculative, since their function(s) is unclear. However, should these polypeptides constitute an auxin-specific uptake symport, then no modifications of current models would be necessary (see REVIEW OF LITERATURE), since it is

possible to envision auxin transport and auxin perception as occurring by separate mechanisms. In fact, in this circumstance there is no particular reason that an auxin receptor related to growth must be located at the PM. As proposed by Hager et al. (1991), auxin could act directly at the ER to stimulate lipid and wall deposition and at the nucleus to stimulate the specific transcription of genes such as those of the PM H<sup>+</sup>-ATPase via soluble auxin receptors.

Should the axido-IAA labeled polypeptides be more strongly implicated in auxin-induced growth, any model would become more complicated. It would have to account for the ER-localized 22 kDa auxin-binding protein and its potential involvement in growth. A mechanism involving the coupling of a 40 kDa and 42 kDa PM receptor to an ER-localized receptor, such as that of Klæmbt (1990), would seem unlikely, since both the PM and ER proteins directly bind auxin. Therefore, it would seem more likely that the two receptors would control different aspects of the growth process. In such a model, the ER receptor could modulate exocytosis of lipid and wall components, while the PM receptor could be coupled via a signal transduction chain to transcription factors which control specific gene expression. The nature of the transduction chain is unclear, but classical components of eukaryotic signal transduction systems such as phosphatidylinositol metabolism (Ettlenger and Lehle, 1988); Scherer and Andre, 1989), G-proteins (Ma et al., 1990) and receptor kinases (Walker and Zhang, 1990) have been found in plants.



Much effort has been applied toward obtaining amino acid sequence data from the azido-IAA binding proteins. Partial sequence data is available, but it is insufficient for designing oligonucleotide probes or a database search. When cDNAs become available, it will be possible to determine the cellular function(s) of the PM 40 kDa and 42 kDa azido-IAA binding proteins and to examine in detail their mode of action at the molecular level.

## VII. BIBLIOGRAPHY

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989) Short Protocols in Molecular Biology (Wiley, New York), pp. 328.
- Barbier-Brygoo, H., Edphritikhine, G., Klæmbt, D., Ghislan, M., and Guern, J. (1989) Functional evidence for an auxin receptor at the plasmalemma of tobacco mesophyll protoplasts. Proc. Natl. Acad. Sci, USA 86, 891-895.
- Barbier-Brygoo, H., Edphritikhine, G., Shen, W.H., Delbarre, A., Klæmbt, D., and Guern, J. (1990) Characterization and modulation of sensitivity of plant protoplasts to auxin. in, Activation and Desensitization of Transducing Pathways, NATO ASI Series, Series H, Cell Biology, Secaucus, NJ, Vol. 44, eds. Konijn, T.M., Houslay, M.D. and Van Haastertm P,J,M, (Springer-Verlag, Heidelberg), pp. 231-244.
- Bayley, H. and Knowles, J.R. (1977) Photoaffinity labeling. Methods Enzymol. 46, 69-114.
- Benning, C. (1986) Evidence supporting a model of voltage-dependent uptake of auxin into *Cucurbita* vesicles. Planta 169, 228-237.
- Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256(4), 1604-1607.
- Bradford, K.J. and Yang, S.F. (1980) Stress-induced ethylene production in the ethylene-requiring tomato mutant *diageotropica*. Plant Physiol. 65, 327-330.
- Chowdhry, V. and Westheimer, F.H. (1979) Photoaffinity labeling of biological systems. Annu. Rev. Biochem. 48, 293-325.
- Clause, S. and Rayle, D.L. (1991) Auxin induction of a SAUR homolog in tomato and its absence in an auxin-insensitive mutant. ASGSB Bulletin 5(1) 76.
- Cleland, R.E. (1973) Auxin-induced hydrogen ion secretion from *Avena* coleoptiles. Proc Natl. Acad. Sci., USA 70, 3092-3093.
- Cleland, R.E. (1976) Kinetics of hormone-induced H<sup>+</sup>-secretion. Plant Physiol. 58, 210-213.

- Cross, J.W. (1985) Auxin action: The search for the receptor. *Plant Cell Environ.* 8, 352-359.
- Cross, J.W. (1991) Cycling of auxin-binding protein through the plant cell: Pathways in auxin signal transduction. *The New Biologist* 3(8), 1-7.
- Cross, J.W. and Briggs, W.R. (1978) Properties of a solubilized microsomal auxin-binding protein from coleoptiles and primary leaves of *Zea mays*. *Plant Physiol.* 62, 152-157.
- Daniel, S.G., Rayle, D.L. and Cleland, R.E. (1989) Modification of polar auxin transport by ethylene in the auxin-insensitive tomato mutant *diageotropica*. *Plant Physiol.* 91, 804-807.
- Darwin, C. (1880) *The power of movement in plants* (Murry, London).
- Davies, P.J. (1987) *Plant Hormones and Their Role in Plant Growth and Development*, ed. Davies, P.J. (Martinus Nijhof, Dordrecht).
- Dohrmann, U., Hertel, R. and Kowlik, H. (1978) Properties of auxin binding sites in different subcellular fractions from maize coleoptiles. *Planta* 140, 97-106.
- Dunbar, B.S. (1987) *Two-Dimensional Electrophoresis and Immunological Techniques* (Plenum, New York), pp. 47-65.
- Ettlinger, C. and Lehle, L. (1988) Auxin induces rapid changes in phosphatidylinositol metabolites. *Nature* 331, 176-178.
- Evans, M.L. and Vesper, M.J. (1980) An improved method for detecting auxin-induced hydrogen ion efflux from corn coleoptile segments. *Plant Physiol.* 66, 561-565.
- Fujino, D.W., Nissen, S.J., Jones, A.D., Burger, D.W., and Bradford, K.J. (1988) Quantification of indole-3-acetic acid in dark-grown seedlings of the *diageotropica* and *epinastic* mutants of tomato (*Lycopersicon esculentum* Mill.) *Plant Physiol.* 88, 780-784.
- Goldsmith, M.H.M. (1977) Polar auxin transport, *Ann. Rev. Plant Physiol.* 28, 439-478.
- Hagen, G. (1989) Molecular approaches to understanding auxin action. *The New Biologist* 1(1), 19-23.

- Hager, A., Debus, G., Edel, H-G., Strabsky, H., and Serrano, R. (1991) Auxin induces exocytosis and the rapid synthesis of a high-turnover pool of plasma-membrane H<sup>+</sup>-ATPase. *Planta* 185, 527-537.
- Hager, A., Menzel, H. and Krauss, A. (1971) Versuche und hypothese zur primarwirkung des auxins beim streckungswachstum. *Planta* 100, 47-75.
- Hames, B.D. (1981) Gel Electrophoresis of Proteins, Hames, B.D. and Rickwood, D., eds. (IRL Press, Oxford), pp. 44-45.
- Hertel, R. (1986) Two comments on auxin transport: The uptake/efflux mechanism and the problem of adaptation. in, *Plant Growth Substances 1985*, ed. Bopp, M. (Springer-Verlag, Heidelberg), pp. 214-217.
- Hertel, R. and Leopold, A.C. (1963) Versuche zur analyse des auxintransports in der koleoptile von *Zea mays* L. *Planta* 59, 535-562.
- Hertel, R., Lomax, T.L. and Briggs, W.R. (1983) Auxin transport in membrane vesicles from *Cucurbita pepo* L. *Planta* 157, 193-201.
- Hertel, R., Thomson, K-St. and Russo, V.E.A. (1972) In-vitro binding to particulate cell fractions from corn coleoptiles. *Planta* 107, 325-340.
- Hesse, T., Feldwisch, J., Balshusemann, D., Bauw, G., Puype, M., Vandekerckhove, J., Løbler, M., Klæmbt, D., Schell, J., Palme, K. (1989) Molecular cloning and structural analysis of a gene from *Zea mays* (L.) coding for a putative receptor for the plant hormone auxin. *EMBO J.* 8(9), 2453-2461.
- Heukeshoven, J. and Dernick, R. (1985) Simplified method for silver staining. *Electrophoresis* 6, 103-112.
- Inohara, N., Shimomura, S., Fukui, T., Futai, M. (1989) Auxin-binding protein located in the endoplasmic reticulum of maize shoots: Molecular cloning and complete primary sequence. *Proc. Natl. Acad. Sci., USA* 86, 3564-3568.
- Jackson, M.B. (1979) Is the *diageotropica* tomato ethylene deficient? *Physiol. Plant.* 46, 347-351.

- Jackson, M.B. and Campbell, D.J. (1975) Movement of ethylene from roots to shoots, a factor in the responses of tomato plants to waterlogged soil conditions. *New Phytol.* 74, 397-406.
- Jacobs, M. and Gilbert, S.F. (1983) Basal localization of the presumptive auxin transport carrier in pea stem cells. *Science* 220, 1297-1300.
- Jacobs, M. and Hertel, R. (1978) Auxin binding to subcellular fractions from *Cucurbita* hypocotyls: In vitro evidence for an auxin transport carrier. *Planta* 142, 1-10.
- Jacobs, M. and Ray, P. (1976) Rapid auxin-induced decrease in free space pH and its relationship to auxin-induced growth in maize and pea. *Plant Physiol.* 58, 203-209.
- Jacobs, M. and Rubery, P.H. (1988) Naturally occurring auxin transport regulation. *Science* 241, 346-349.
- Jacobs, M. and Short, T.W. (1986) Further characterization of the presumptive auxin transport carrier using monoclonal antibodies. in, *Plant Growth Substances 1985*, ed. Bopp, M. (Springer-Verlag, Heidelberg), pp. 218-226.
- Jacobs, M. and Short, T.W. (1987) Preliminary separation of pea stem NPA receptors by high performance ion exchange chromatography. in, *Plant Hormone Receptors*, ed. Klæmbt, D. (Springer-Verlag, Heidelberg), pp. 93-97.
- Jacobs, M. and Taiz, L. (1980) Vanadate inhibition of auxin-enhanced H<sup>+</sup> secretion and elongation in pea epicotyls and oat coleoptiles. *Proc. Natl. Acad. Sci., USA* 77, 7242-7246.
- Jones, A.M. (1990a) Do we have an auxin receptor yet? *Physiol. Plant.* 80, 1540-158.
- Jones, A.M. (1990b) Location of transported auxin in etiolated maize shoots using 5-azidoindole-3-acetic acid. *Plant Physiol.* 93, 1154-1161.
- Jones, A.M., Melhado, L.L., Ho, T-H. D., and Leonard, N.J. (1984a) Azido auxins. Quantitative binding data in maize. *Plant Physiol.* 74, 295-301.
- Jones, A.M., Melhado, L.L., Ho, T-H. D., Pearce, C.J., and Leonard, N.J. (1984b) Azido auxins. Photoaffinity labeling of auxin-binding proteins in maize coleoptile with tritiated 5-azidoindole-3-acetic acid. *Plant Physiol.* 75, 1111-1116.

- Jones, A.M. and Venis, M.A. (1989) Photoaffinity labeling of indole-3-acetic acid-binding protein in maize. Proc. Natl. Acad. Sci., USA 86, 6153-6156.
- Jones, A.M. and Venis, M.A. (1988) Physiology and Biochemistry of Auxins in Plants, eds., Kutachek, M., Bandurski, R.S., and Krekule, J. (Academia, Prague) pp. 165-166.
- Kehry, M.R. and Dahlquist, F.W. (1982) The methyl-accepting chemotaxis proteins of *Escherichia coli*: Identification of the multiple methylation sites on methyl-accepting chemotaxis protein. J. Biol. Chem. 257, 10378-10386.
- Kelly, M.O. and Bradford, K.J. (1986) Insensitivity of the *diageotropica* tomato mutant to auxin. Plant Physiol. 82, 713-717.
- Kikuchi, M., Imasei, H. and Sakai, S. (1989) Modulation of gene expression in isolated nuclei by auxin-binding proteins. Plant Cell Physiol. 30, 765-773.
- Klæmbt, D. (1990) A view about the function of auxin-binding proteins at plasma membranes. Plant Mol. Biol. 14, 1045-1050.
- Klekamp, M.S. and Weil, P.A. (1987) The properties of yeast class III transcription factor TFIIIB. J. Biol. Chem. 262(16), 7878-7883.
- Larsson, C. (1985) Plasma membranes. in, New Series, vol. 1: Cell Components, eds. Haskins, H.F. & Jackson, J.F. (Springer, Berlin), pp. 85-104.
- Libbenga, K.R. and Mennes, A.M. (1987) Hormone binding and its role in hormone action. in, Plant Hormones and Their Role in Plant Growth and Development, ed. Davies, P.J. (Nijhoff, Dordrecht), pp. 194-221.
- Løebler, M. and Klæmbt, D. (1985a) Auxin binding protein from coleoptile membranes of corn (*Zea mays* L.) I. Purification by immunological methods and characterization. J. Biol. Chem. 9848-9853.
- Løebler, M. and Klæmbt, D. (1985b) Auxin binding protein from coleoptile membranes of corn (*Zea mays* L.) II. Localization by immunological methods and characterization. J. Biol. Chem. 260, 9854-9859.

- Lomax, T.L. (1986) Active auxin uptake by specific plasma membrane carriers. in, *Plant Growth Substances 1985*, ed. Bopp, M. (Springer, Berlin), pp. 209-213.
- Lomax, T.L., Conley, P.B., Shilling, J., and Grossman, A.R. (1987) Isolation and characterization of light-regulated phycobilisome linker polypeptide genes and their transcription as a polycistronic mRNA. *J. Bact.* 169, 2675-2684.
- Lomax, T.L. and Hicks, G.R. (1992) Specific auxin-binding proteins in the plasma membrane: Receptors or transporters? *Biochem. Soc. Trans.* 20, 64-69.
- Lomax, T.L., Melhorn, R.J. and Briggs, W.R. (1985) Active auxin uptake by zucchini membrane vesicles and quantitation using ESR volume and  $\Delta$ pH determinations. *Proc. Natl. Acad. Sci., USA* 82, 6541-6545.
- Lord, J.M. (1983) Isolation of Membranes and Organelles from Plant Cells, eds., Hall, J.L. and Moore, A.L. (Academic Press, London), pp. 119-134.
- Luthen, H., Bigdon, M. and Bottger, M. (1990) Re-examination of the acid growth theory of auxin action. *Plant Physiol.* 93, 931-939.
- Ma, H., Yanofsky, M.F. and Meyerwitz, E.M. (1990) Molecular cloning and characterization of GPA1, a G protein alpha subunit from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci., USA* 87, 3821-3825.
- Marre, E. (1979) Fusicoccin: A tool in plant physiology. *Annu. Rev. Plant Physiol.* 30, 273-288.
- McClure, B.A. and Guilfoyle, T.J. (1987) Characterization of a class of small auxin-inducible polyadenylated RNAs. *Plant Mol. Biol.* 9, 611-623.
- McClure, B.A. and Guilfoyle, T.J. (1989a) Rapid redistribution of auxin-regulated RNAs during gravitropism. *Science* 243, 91-93.
- McClure, B.A. and Guilfoyle, T.J. (1989b) Tissue print hybridization. A simple technique for detecting organ- and tissue-specific gene expression. *Plant Mol. Biol.* 12, 517-524.
- McDonald, H., Jones, A.M. and King, P. (1991) Photoaffinity labeling of soluble auxin-binding proteins. *J. Biol. Chem.* 266(12), 7393-7399.

- Melhado, L.L., Jones, A.M., Leonard, N.J. and Vanderhoef, L.N. (1981) Azido auxins: Synthesis and biological activity of fluorescent photoaffinity labeling agents. *Plant Physiol.* 68, 469-475.
- Mentze, J., Raymond, B., Cohen, J.D., and Rayle, D.L. (1977) Auxin-induced H<sup>+</sup>-secretion in *Helianthus* and its implications. *Plant Physiol.* 60, 509-512.
- Munro, S. and Pelham, H.R.B. (1987) A C-terminal signal prevents the secretion of ER luminal proteins. *Cell* 48, 899-907.
- Nagahashi, J. & Kane, A.P. (1982) Triton-stimulated nucleoside diphosphatase activity: Subcellular localization in corn root homogenates. *Protoplasma* 112, 167-173.
- Napier, R.M. and Venis, M.A. (1990) Receptors for plant growth regulators: Recent advances. *J. Plant Growth Regul.* 9, 113-126.
- O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007-4021.
- Palme, K., Brzobohaty, B., Feldwisch, J., Garbers, C., Hesse, F., Hesse, T., Moore, T., Walden, R., Zettl, R., and Schell, J. (1991a) in, A molecular approach to study the perception of the auxin signal. in, Abstracts 14th International Conference on Plant Growth Substances, Amsterdam, July 21-26, pp. 36.
- Palme, K., Hesse, T., Moore, I., Campos, N., Feldwisch, J., Garbers, C., Hesse, F., and Schell, J. (1991b) Hormonal modulation of plant growth: The role of auxin perception. *Mech. of Dev.* 33, 97-106.
- Palme, K. and Schell, J. (1991) Auxin receptors take shape. *Current Biology* 4, 228-230.
- Perbal, G., Driss-Ecole, D. and Leroux, Y. (1982) Basipetal transport of [<sup>3</sup>H]IAA in the wheat coleoptile. *Physiol. Plant.* 56, 285-294.
- Prasad, P.V. and Jones, A.M. (1991) Putative receptor for the plant hormone auxin identified and characterized by anti-idiotypic antibodies. *Proc. Natl. Acad. Sci., USA* 88, 5479-5483.
- Rayle, D.L. (1973) Auxin-induced hydrogen ion excretion in *Avena* coleoptiles and its implications. *Planta* 114, 63-73.



- Rayle, D.L. and Cleland, R.E. (1970) Enhancement of wall loosening and elongation by acid solutions. *Plant Physiol.* 46, 250-253.
- Ripp, K.B., Viitanen, P.V., Hitz, W.D., and Franceschi, V.R. (1988) Identification of a membrane protein associated with sucrose transport into cells of developing soybean cotyledons. *Plant Physiol.* 88, 1435-1445.
- Rubery, P.H. (1981) Auxin receptors. *Ann. Rev. Plant Physiol.* 32, 569-596.
- Rubery, P.H. and Sheldrake, A.R. (1974) Carrier-mediated auxin transport. *Planta* 118, 101-121.
- Ruoho, A., Rashidbaigi, A., and Roeder, P. (1984) Approaches to the identification of receptors utilizing photoaffinity labeling. in, *Receptor Biochemistry and Methodology, Volume 1*, eds. Venter, C.J., Harrison, L.C. (Alan R. Liss, New York), pp. 119-160.
- Sabater, M. and Rubery, P.H. (1987a) Auxin carriers in *Cucurbita* vesicles. II. Evidence that carrier-mediated routes of both indole-3-acetic acid influx and efflux are electroimpelled. *Planta* 171, 507-513.
- Sabater, M. and Rubery, P.H. (1987b) Auxin carriers in *Cucurbita* vesicles. III. Specificity, with particular reference to 1-naphthaleneacetic acid. *Planta* 171, 514-518.
- Sakai, S. and Hanagata, T. (1983) Purification of an auxin-binding protein from etiolated mung bean seedling by affinity chromatography. *Plant Cell Physiol.* 24, 685-693.
- Sandstrom, R.P., de Boer, A.H., Lomax, T.L., and Cleland, R.E. (1987) Latency of plasma membrane H<sup>+</sup>-ATPase in vesicles isolated by aqueous phase partitioning. *Plant Physiol.* 85, 693-698.
- Scherer, G.F.E. and Andre, B. (1989) A rapid response to a plant hormone: Auxin stimulates phospholipase A<sub>2</sub> in vivo and in vitro. *Biochem. Biophys. Res. Comm.* 163, 111-117.
- Shimomura, S., Inohara, N., Fukui, T., and Futai, M. (1988) Different properties of two types of auxin-binding sites in membranes from maize coleoptiles. *Planta* 175, 558-566.
- Shimomura, S., Stobayashi, T., Futai, M., and Fukui, T. (1986) Purification and properties of an auxin-binding protein from maize shoot membranes. *J. Biochem.* 99, 1513-1524.

- Spector, T. (1987) Refinement of Coomassie blue method of protein quantitation. *Anal. Biochem.* 86, 142-146.
- Taiz, L. and Zeiger, E. (1991) *Plant Physiology* (Benjamin/Cummings, Redwood City), pp. 407-411.
- Tillmann, U., Viola, G., Kayser, B., Siemeister, G., Hesse, T., Palme, K., Løebler, M., and Klæmbt, D. (1989) cDNA clones of the auxin-binding protein from corn coleoptiles (*Zea mays* L.): Isolation and characterization by immunological methods. *EMBO J.* 8(9), 2463-2467.
- Tolbert, N.E. (1974) Isolation of subcellular organelles of metabolism on isopycnic sucrose gradients. *Methods Enzymol.* 31, 734-746.
- Ursin, V. and Bradford, K.J. (1989) Unique phenotype in heterozygotes of the auxin-insensitive mutant of tomato, *diageotropica*. *Plant Physiol.* 90, 1241-1245.
- Venis, M.A. (1980) Purification and properties of membrane-bound auxin receptors in corn. in, *Plant Growth Substances 1979*, ed. F. Skoog (Springer, Berlin) pp. 61-70.
- Voet, J.G., Howley, K.S. and Shumsky, J.S. (1987) 5-azido-N-1-naphthylphthalamic acid, a photolabel analog of N-1-naphthylphthalamic acid. Synthesis and binding properties in *Cucurbita pepo* L. *Plant Physiol.* 85, 22-25.
- Walker, J.C. and Key, J.L. (1982) Isolation of cloned cDNAs to auxin-responsive poly (A)<sup>+</sup> RNAs of elongating soybean hypocotyl. *Proc. Natl. Acad. Sci., USA* 79, 7185-7189.
- Walker, J.C. and Zhang, R. (1990) Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica*. *Nature* 345, 742-746.
- Went, F.W. (1928) Wuchstoff und wachstum. *Rec. Trav. Bot. Neerl.* 25, 1-1.
- Wightman, F., Schneider, E.A. and Thimann, K.V. (1980) Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in Alaska peas roots. *Physiol. Plant.* 49, 304-314.

- Wright, A.D., Simpson, M.B., Neuffer, M.G., Michalezuk, L., Slovin, J.P., and Cohen, J.D. (1991) Indole-3-acetic acid biosynthesis in the mutant maize *orange pericarp*, a tryptophan auxotroph. *Science* 254, 998-1000.
- Zobel, R.W. (1969) The genetics and development of the tomato mutant *lazy 3 (lz-3)*. MS thesis. University of California, Davis.
- Zobel, R.W. (1972a) Genetics and physiology of two root mutants in tomato *Lycopersicon esculentum* Mill. PhD thesis. University of California, Davis.
- Zobel, R.W. (1972b) Genetics of the *diageotropica* mutant in the tomato. *J. Hered.* 63, 94-97.
- Zobel, R.W. (1973) Some physiological characteristics of the ethylene-requiring tomato mutant *diageotropica*. *Plant Physiol.* 52, 385-389.
- Zobel, R.W. (1974) Control of morphogenesis in the ethylene-requiring tomato mutant, *diageotropica*. *Can. J. Bot.* 52, 735-741.