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Auxin-induced growth of peeled and unpeeled stem segments of the wildtype and Argenteum varieties of *Pisum sativum*

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**AUXIN-INDUCED GROWTH
OF PEELED AND UNPEELED STEM SEGMENTS
OF THE WILDTYPE AND ARGENTEUM VARIETIES OF *PISUM SATIVUM***

A Thesis

Presented to

The Faculty of the Department of Biology

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

by

Donna Garvey Brickner

May 1995

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ABSTRACT

AUXIN-INDUCED GROWTH OF PEELED AND UNPEELED STEM SEGMENTS OF THE WILDTYPE AND ARGENTEUM VARIETIES OF *PISUM SATIVUM*

by Donna Garvey Brickner

The epidermis has been considered the sole target tissue for auxin based on the lack of auxin-induced growth in peeled stem segments. These experiments did not take into account the possibility of a wound response inhibiting growth. In this study, experiments with a mutant pea variety, *argenteum*, revealed the occurrence of auxin-induced growth without an epidermis. Investigation by brightfield, fluorescence, and electron microscopy did not reveal secondary wall deposition in response to peeling in any experimental treatment. Nevertheless, exocytosis of Golgi-derived vesicles was prominent, indicative of initial stages of secondary wall deposition that accompanies wound repair. An acid phosphatase reaction was used to localize enzymatic activity concurrent with a wound response. In unpeeled wildtype and peeled mutant segments, the reaction was typical of non-senescing cells, whereas peeled wildtype segments showed activity typical of a cell undergoing a wound reaction.

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CHAPTER ONE

INTRODUCTION

Multicellular organisms possess dynamic and complex modes of intercellular communication. Endocrine, paracrine, and autocrine signals in animals regulate differentiation, excitation, and growth in a coordinate manner. The most prevalent signaling molecules in plants and animals are hormones. The term, hormone, refers in animals to an endocrine signaling molecule in a secretory gland that is delivered to the target tissue via the circulatory system. Extremely small amounts of these molecules are necessary and typically influence a specific physiological process. Hormones that stimulate cell proliferation in animal systems include epidermal growth factor and platelet-derived growth factor (for review, see Voet and Voet, 1990). Plant hormones differ from animal hormones in that the site of synthesis is not always clearly delineated from the site of action, although plant hormones also often elicit responses at sites distant from their synthesis. Whereas animal hormones tend to elicit specific biochemical responses, plant hormones can effect multiple and diverse responses depending on the target organ or tissue (for review, see Wareing and Phillips, 1978).

Hormone-Mediated Processes

The most well characterized classes of hormones, which regulate a wide variety of cellular processes in plants, are auxins, cytokinins, and gibberellins. Additionally, other classes of plant hormones exist that are generally growth

inhibitors, such as abscisic acid (ABA) and ethylene. Auxins promote the elongation of excised but rarely intact stem and root segments, the formation of adventitious roots, and the inhibition of lateral bud formation. Cytokinins promote cell division, delay senescence, and perhaps increase the translocation of nutrients to developing tissues. They also promote lateral bud break and cell expansion in dicot cotyledons and leaves and stimulate the development of chloroplasts from etioplasts and proplastids. Gibberellins, 84 of which have been isolated, promote internodal extension in the intact plant, overcome seed and bud dormancy, invoke flowering, and circumvent the need for many inductive environmental factors, such as a critical light requirement or an inductive cold period. ABA heightens responses that allow plants to cope with environmental stresses, such as water deficits, saline soils, low temperature, and frost. ABA also facilitates normal embryogenesis and formation of seed-storage proteins and prevents premature germination and growth of many seeds and buds. Ethylene, the only known gaseous hormone, promotes fruit ripening, uniform flowering, leaf abscission, and senescence. It inhibits pea stem elongation, induces stem thickening, and retards epicotyl hook opening but also promotes adventitious root formation (for review, see Salisbury and Ross, 1992).

Auxin-Induced Growth

Aerial plant organs, such as stems and coleoptiles, grow by plastic cell enlargement; cell growth can be defined as the irreversible expansion of the cell due to the force of turgor pressure. This expansion allows increased water uptake into the vacuole which in turn increases cell volume. Due to the

anisotropic nature of plant cells, they tend to enlarge in length while remaining a constant width (Fosket, 1994). The properties of cell growth have been the focus of many plant physiologists' work for many decades, and yet many subtle questions remain unanswered.

Since Went's discovery of auxin in the 1920s, plant physiologists have studied the growth hormone's mode of action and its effect on excised plant tissues. Auxin supplied exogenously to internodal or coleoptile segments causes elongation. Auxin causes individual parenchyma cells to expand by a mechanism involving wall acidification, which promotes wall loosening, followed by transcription and translation of growth-mediating enzymes and structural proteins. The target tissue for auxin has been reported to be exclusively the epidermis.

There are a number of possibilities for auxin's mode of action. It may bind to an auxin receptor on the plasma membrane and initiate a signal-transduction cascade that stimulates a membrane-bound H^+ -ATPase (H^+ pump) that acidifies and loosens the wall (Barbier-Brygoo *et al.*, 1991; Venis *et al.*, 1992). Through the same or similar transduction pathway, activation of cellular or extracellular enzymes may occur, some of which may be involved in the growth process. On the other hand, auxin might enter the cell and bind to an internal, soluble, cytoplasmic receptor, similar to steroid hormone action in animal cells. This ligand receptor complex would then migrate into the nucleus as a transcription factor (for review, see Fosket, 1994; Sakai, 1992; MacDonald *et al.*, 1991). This kind of cellular response would promote growth through increased transcription of specific mRNAs and specific growth-mediating enzymes. Alternatively, auxin may have multiple target sites,

some on the cell surface and others within the cell. Binding to the cell-surface receptor may trigger only proton secretion, whereas binding to an endoplasmic-reticulum or soluble cytoplasmic receptor may stimulate the processing of wall precursors and their secretion into the wall as well as the synthesis of a low pH-optimum, wall-building enzyme.

Typically auxin-mediated growth responses have been investigated in isolated tissue incubated with an auxin or auxin-like growth regulator present. It is important to note that experiments with excised stem segments might not be representative of processes occurring in the intact plant (Brummell and Hall, 1987). Hanson and Trewavas (1982) argued that application of auxin to intact plants failed to elicit a significant growth response. Work on etiolated pea (Hall *et al.*, 1985), sunflower and marrow (Tamimi and Firn, 1985), and watermelon (Carrington and Esnard, 1988) showed strong, albeit transient, auxin-induced stem elongation; after five hours of elongation, auxin became inhibitory. In contrast, more recent experiments on both dwarf and tall varieties of the garden pea demonstrated that auxin strongly promoted long-term stem elongation in intact plants of that species (Yang *et al.*, 1993). Indole-3-acetic acid (IAA) was supplied continuously at a high concentration (100 μ M) to the topmost internodes of the plant via a cotton wick. The growth promotion was particularly pronounced in the dwarf variety, which grew six times more than control seedlings over a prolonged period.

Gene-Activation Hypothesis

Auxin-induced growth involves the loosening of growth-limiting cell walls. The actual mechanism is still unknown, but three main hypotheses

have been put forth, more than one of which may be operating simultaneously. First, in the gene-activation hypothesis proposed by many investigators in the 1960s, auxin activates genes encoding for the proteins necessary for growth (Masuda *et al.*, 1967; Theologis, 1986). Growth inhibition in the presence of RNA or protein synthesis inhibitors is consistent with this hypothesis (Barkley and Evans, 1970; Theologis, 1986). In an earlier approach, Masuda *et al.* (1967) measured the incorporation of radiolabeled uracil and found that IAA stimulates the biosynthesis of RNA in oat coleoptiles. It was assumed at that time that this was mRNA.

Trewavas (1968) observed an increase in the number of polysomes present in etiolated pea seedlings treated with IAA, indicating that ribosomal RNA, but not mRNA, synthesis is stimulated by the hormone. Pope and Black (1972) found that even with treatment with cyclohexamide, a protein synthesis inhibitor, IAA stimulates extension of wheat coleoptiles, implying that the structural proteins and growth-mediating enzymes detected are from a previously synthesized pool and their turnover is slow. Penny *et al.* (1972) also found that auxin-induced growth occurs in lupine hypocotyl and pea epicotyl segments treated with the transcription inhibitor, actinomycin D. Furthermore, elongation-specific mRNAs localized in the epidermis of etiolated peas do not appear until at least one hour after incubation in IAA despite the occurrence of growth (but perhaps acid-induced growth only) within 15 minutes (Dietz *et al.*, 1990). These data support the conclusion that RNA synthesis is not necessary for the initial stages of auxin-induced elongation but is necessary to maintain the growth response for extended periods.

Acid-Growth Hypothesis

The second hypothesis for the mode of action of auxin in stem elongation is that the hormone acidifies the cell-wall matrix, bringing about wall loosening and elongation (Rayle and Cleland, 1970). This hypothesis is appealing, because auxin is known to induce the secretion of protons into the extracellular matrix, acidifying the bathing medium. Furthermore, tissue segments placed in acidic incubation media ($\text{pH} < 4$) elongate (Cleland and Rayle, 1970; Hager *et al.*, 1971). However, the relationship between proton secretion and auxin-induced growth is not necessarily causative. Kutchera and Schopfer (1985b) provided evidence against the acid-growth hypothesis by abrading maize coleoptiles. In their research, experimental wall acidification to a pH of 3.5-4.0 generates coleoptile growth comparable to that generated by auxin alone. However, growth is absent at pH 4.5-5.0, the range reported to result from the exogenous application of auxin (Rayle and Cleland, 1977). In addition, buffering the wall during auxin exposure to prevent wall acidification does not prevent long-term, auxin-induced growth (Kutchera and Schopfer, 1985b). They, therefore, concluded that wall acidification and growth are mechanistically unrelated (Kutchera and Schopfer, 1985a, b; Kutchera *et al.*, 1987; Schopfer, 1989). Other researchers have found that light-grown peas are responsive to auxin but not to a low pH (Barkley and Leopold, 1973). On the other hand, Cleland *et al.* (1991) reported that, in peeled *Avena* coleoptiles incubated in a buffer at pH 5, elongation was comparable to that of auxin-induced growth. They contended that acid-induced growth is still a viable hypothesis for sufficiently abraded plant tissue ($>50\%$).

A plausible mechanism to account for the growth of stem segments in response to the secretion of protons into the apoplast and a low wall pH is that an acid environment is necessary for optimal functioning of a wall-loosening enzyme. Auxin is proposed to bind to and stimulate the activity of a membrane-bound H⁺-ATPase (Fosket, 1994). Protons are subsequently secreted into the cell-wall matrix, reducing the pH to the optimum for an enzyme that breaks the H⁺ bonds between xyloglucans and cellulose microfibrils. This wall loosening is essential for growth and occurs rapidly (within 15 minutes) in response to the hormone (Fosket, 1994).

Incorporation of Cell-Wall Material

The third hypothesis to explain auxin-induced growth proposes a causal relationship between the production of cell-wall materials and the degree of elongation (Ray, 1962). In pea stem segments, auxin induces the rapid synthesis of noncellulosic polysaccharides in the epidermis (Kutcher and Briggs, 1987). These studies support the hypothesis that auxin induces growth by a rapid stimulation of the secretion and incorporation of wall material in the elongating tissue. However, Edelman (1989) reported that the apposition of cellulose microfibrils occurs independently of IAA-induced elongation, responding secondarily to hemicellulosic wall deposition but not, by itself, limiting the rate of wall stretching. These conflicting results can be reconciled by the fact that the rate of cell expansion is controlled by the incorporation of noncellulosic polysaccharides rather than by cellulose deposition.

Microtubule and Microfibril Reorientation

In addition to affecting the rate and amount of growth, auxin also affects the direction of growth through its influence on cortical microtubule and microfibril orientation. Microfibrils are initially wrapped around the cell in a helical fashion and, as the cell elongates, they become reoriented from shallow to steeply pitched helices (termed transversely and longitudinally oriented, respectively). Rapidly elongating internodes in young seedlings bear mostly transversely oriented microtubules and microfibrils (Laskowski, 1990; Takeda and Shibaoka, 1982), whereas older, senescing cells and cells devoid of auxin bear predominately longitudinally oriented microtubules (Laskowski, 1990). Upon exposure to auxin, microtubules oriented in a longitudinal fashion reorient to a transverse direction in radish hypocotyls (Sakoda *et al.*, 1992) and in sunflower hypocotyls and maize coleoptiles (Bergfeld *et al.*, 1988; Nick *et al.*, 1990; Zandomeni and Schopfer, 1993). Ethylene also affects the orientation of microtubule and microfibril deposition. Lang *et al.* (1982) found that the cortical microtubules of pea epicotyls treated with ethylene are primarily longitudinal. The inability to reorient microfibrils to the transverse direction would explain the absence of elongation and presence of radial cell expansion, or swelling, in ethylene-treated stem segments. It is also consistent with the notion that auxin promotes elongation rather than uniform cell expansion by directly or indirectly reorienting microfibrils.

Auxin Receptors

Auxin-binding proteins (ABP) that are putative receptors have been identified in plants. As the term “receptor” implies, it is assumed that biochemical action follows binding of the ligand to bring about some physiological response. For an auxin-binding protein to qualify as a receptor, it must reversibly bind the hormone with high affinity at physiological concentrations, be saturable, and not bind analogs that lack auxin activity. Several ABPs have been isolated and purified (Löbner and Klämbt, 1985a; Prasad and Jones, 1991; Venis *et al.*, 1992). When antibodies against the functional domains of these proteins were added to the medium surrounding an excised stem or coleoptile segment, competitively binding the ABPs, auxin-induced elongation was prevented (Löbner and Klämbt, 1985b; Prasad and Jones, 1991; Hoson *et al.*, 1992). This result is typically considered sufficient evidence that the protein is an auxin receptor. There are reportedly three different intracellular sites for auxin binding in corn coleoptiles. Site I is located in the endoplasmic reticulum (ER), site II at the tonoplast, and site III at the plasmalemma (Rubery, 1981; Löbner and Klämbt, 1985a; Palme, 1993).

One intensely studied ABP, and the one most widely accepted as a true auxin-receptor, is apparently a homodimer of two polypeptides, each of about 22 kDa (Löbner and Klämbt, 1985a; Brzoboharty *et al.*, 1993). The receptor, ABP1 from maize, has been characterized (Löbner and Klämbt, 1985a; Venis *et al.*, 1992) and determined to be similar to the site I receptor. Approximately 80-90% of ABP1 is localized in the lumen of the ER. This protein has been sequenced from cDNA and has a KDEL (K=lysine, D=aspartic acid, E=glutamic acid, L=leucine) sequence that is a known ER-localization signal (Fosket, 1994).

This receptor is also found on the outer surface of the plasma membrane (PM), most likely being presented to the PM after cleavage of the ER-localization signal (Fosket, 1994). It is curious that many report ABP1 to face the extracellular surface of the PM and that impermeable, active auxin analogs cause a transmembrane potential associated with auxin binding to a receptor (Venis *et al.*, 1990).

Löbler and Klämbt (1985b) also showed by immunofluorescence that this receptor is localized specifically in the plasmalemma of the outer epidermis in the corn coleoptile. Antibodies to the ABP's auxin-binding domain greatly reduce auxin-induced growth of both excised and longitudinally split coleoptiles (Löbler and Klämbt, 1985b). These researchers concluded that the epidermis is the target tissue for auxin due to receptor localization. They did concede that there may be auxin receptors in the mesophyll tissue but not in large enough numbers to register a detectable fluorescence signal (Löbler and Klämbt, 1985b). Recently, Klämbt (1994) admitted a misinterpretation of these data and recognizes the likelihood of this receptor occurring in internal tissues as well.

Another receptor was identified using anti-idiotypic antibodies, a technique that has been used to identify several receptors in animal systems but had not been used before in any plant systems. Prasad and Jones (1991) identified a 65 kDa protein that specifically binds auxin in a wide variety of plants. This protein is equally distributed along the length of an etiolated soybean stem segment and is localized in cytoplasmic and nuclear fractions. This evidence suggests that this ABP has a direct effect on gene expression by interacting with transcription regulatory elements on the genomic DNA (Prasad and Jones,

1991). This scenario is reminiscent of the steroid-receptor family in animals (for review, see Stryer, 1988).

The details of the signal-transduction pathway triggered by auxin are largely unknown. There is evidence that the hormone's activity at the plasma membrane causes a change in the metabolism of phosphoinositides (Moore *et al.*, 1983; Singh and Paleg, 1986). A phosphatidyl inositol cascade is triggered by hormone binding to surface receptors in animals and may occur in an analogous fashion in plants (Ettinger and Lehle, 1988; Salisbury and Ross, 1992; Scherer and André, 1993; Scherer *et al.*, 1993). The binding of the hormone triggers the cascade, ultimately invoking a wide array of cellular responses (Fig. 1). In many cells, the cascade begins with the activation of a G-protein that in turn activates phospholipase C (PLC). The hydrolysis of the membrane lipid, phosphatidyl inositol-4,5-bisphosphate, by PLC yields two second messengers: inositol trisphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ opens Ca²⁺ channels located in the ER membrane or the tonoplast of the central vacuole, leading to the release of sequestered Ca²⁺ and an increase in the cytosolic Ca²⁺ concentration. The binding of Ca²⁺ to calmodulin results in the further activation of a variety of calmodulin-regulated enzymes. These enzymes have a host of activities, perhaps including processes leading to stem elongation. The other second messenger, DAG, activates members of the protein kinase C family (PKCs), which in turn activate a wide variety of target proteins by phosphorylation of serine and threonine residues. PKCs are important in controlling cell division and proliferation in animal systems (for reviews, see Stryer, 1988; Fosket, 1994) and would be significant in regard to this discussion if they also influence similar processes in plants. Evidence for

their action in plant cells has recently begun to be accumulated (Hansenstein and Evans, 1988; Harper *et al.*, 1991; Schaller *et al.*, 1992).

Role of the Epidermis

Growth regulation in plant stems in response to auxin has long been attributed solely to the epidermis. For more than 50 years, many plant physiologists observed that peeled stem segments lacking an epidermis grow considerably less than unpeeled segments and concluded that the epidermis is the target tissue for auxin. Van Overbeek and Went (1937) showed that removal of the epidermis from pea stem or oat coleoptile segments greatly diminishes the ability of these segments to respond to auxin. Since that time, researchers have performed auxin-induced growth studies on peeled maize coleoptiles, oat coleoptiles, pea epicotyls, and lupine hypocotyls, and all observed a similar phenomenon: peeled segments fail to respond to auxin (Thimann and Schneider, 1938; Penny, 1969; Tanimoto and Masuda, 1971; Masuda and Yamamoto, 1972; Penny *et al.*, 1972; Yamagata *et al.*, 1974; Yamamoto *et al.*, 1974a, 1974b; Brummell and Hall, 1980; Pope, 1982; Kutchera *et al.*, 1987). The epidermis has been proposed to pull the rest of the stem tissue along with it as it expands during exposure to auxin without direct intervention by the hormone in internal regions.

These data do not preclude the possibility that the underlying parenchymatous tissue is capable of a growth response to auxin. Peeling of normal organs cannot be accomplished without wounding the underlying tissue, and epidermal peels are not perfectly clean (Hoch *et al.*, 1980). Because adjacent shared cell walls are tightly adherent and nearly impossible to

separate, cortical cells are invariably ruptured, and remnants adhere to the excised peel. When wounded, plants sense the injury and the underlying cells respond in a number of ways. Suberin or lignin may be laid down in the walls around the trauma site to prevent water loss and viral or bacterial infection (Cutter, 1978; Fahn, 1982). This "closing layer" provides immediate, yet temporary, protection. Suberin and lignin are hard lipoidal compounds that are impermeable to water and thereby seal off the plant's newly exposed walls from the outside environment (Esau, 1977). They are also rigid structural materials that resist elongation. Hence if suberization or lignification occurs in response to the peeling wound, then growth could not occur in peeled segments. Because research on the role of the epidermis in auxin-induced growth has been limited to macroscopic examination of organ length increases over time, a wound response during sample preparation would not be detectable.

Thimann and Schneider (1938) speculated that wounding occurs during the peeling process and that auxin-induced growth in peas lacking an epidermis is reduced by an uncharacterized "damage effect." Kutchera *et al.* (1987) found that the epidermal cells were damaged during the peeling process as indicated by their response to the vital dye, neutral red, and their failure to osmoregulate in hypertonic osmotic solutions. The authors also examined the osmolarity of the buffer solution after peeling and concluded that solutes were not leaking out of wounded surfaces. Other researchers also examined the internal tissues for damage and concluded that the damage was insufficient to interfere with auxin-induced elongation (Pope, 1982). To our

knowledge, neither suberin nor lignin deposition with respect to wounding has been investigated microscopically in peeled pea stem segments.

Acid Phosphatase Localization

Another approach to visualizing a wound response is the detection of the hydrolytic enzyme, acid phosphatase. In nonsenescent plant cells, hydrolases are sequestered within the central vacuole so that normal lytic processes occur without damaging the rest of the cell. When this compartmentalization is eliminated and the hydrolytic enzymes are released, all cytoplasmic constituents are degraded. Numerous functions have been ascribed to acid phosphatases, including the mobilization of food reserves (Yatsu, 1968; Ashford and Jacobsen, 1974), intercellular transport (Braun and Sauter, 1964; Figier, 1968; Gahan and McLean, 1969), and cellular autolysis (Gahan and McLean, 1969; Berjak, 1972; Jones and Villiers, 1972; Matile, 1975). In numerous studies of the subcellular localization of acid phosphatase (*e.g.*, Matile, 1975), acid phosphatase is absent from the cytoplasm of healthy, nonsenescent plant cells. It is anticipated that if a wound response occurred, it would be visualized via the Gomori reaction as an electron-dense precipitate in the cytoplasm. The Gomori reaction is a cytohistochemical technique that, given the proper substrates, creates an electron-dense precipitate in the presence of acid phosphatase, which can then be viewed under an electron microscope.

CHAPTER TWO

The purpose of this work was to determine whether the epidermis is the sole target tissue for auxin in the process of auxin-induced stem segment growth. To test this notion, we investigated the ability of dark-grown, excised stem segments to elongate without an epidermis and the reasons for their failure to elongate. A mutant of the garden pea, *Pisum sativum* var. *argenteum*, was used in a standard straight-growth test. This variety has a single mutation in a gene required for the production of the middle lamella between the epidermis and the mesophyll (Marx, 1978; Hoch *et al.*, 1980). This mutation gives the plant a silvery-gray cast due to air spaces in this region. It also makes the plant easy to peel with little or no damage to the underlying tissues (Hoch *et al.*, 1980; Wilson and Bruck, unpublished data). Stem segments of the mutant and parent varieties were peeled to determine whether growth would occur when a wound response was either eliminated or minimized. The rate of auxin-induced elongation in peeled and unpeeled segments of both varieties was also measured to determine the kinetics of the growth process and whether they were affected by the removal of the epidermis. Another experiment was conducted with an H⁺-pump inhibitor to determine whether the elongation of the peeled segments was a true auxin response and not due to acidification of the medium. Auxin is known to stimulate the activity of an ATPase in the plasma membrane that secretes protons into the cell wall (Fosket, 1994). The H⁺ secretion is believed to be necessary for wall loosening and cell expansion (Fosket, 1994). Additionally, peeled and unpeeled stem segments of the mutant and parent varieties were

examined by transmission electron microscopy for suberization, lignification, acid phosphatase activity and other manifestations of a wound response to explain the lack of growth of unpeeled stem segments used by other investigators.

MATERIALS AND METHODS

Seeds of the mutant of the garden pea (*Pisum sativum* var. *argenteum*) and wildtype seeds (Del Monte Corporation variety #343333) from which the mutant was derived were first soaked in a 10% sodium hypochlorite solution, rinsed several times in distilled water, and then sown in moist vermiculite in wooden flats. The seedlings were grown in a controlled environment chamber under 24 h darkness and 24°C to generate etiolated seedlings. When the seedlings were 10-11 days old, the top-most internode expanded out of the apical bud was removed and used for the experimental treatments. Starting 3 mm below the epicotyl hook, the internode was cut into 0.5 cm segments and placed in the appropriate solution, as described below. Growth was measured with an ocular micrometer under a Wild stereomicroscope.

When peeling a stem segment, the following procedure was used to reduce injury. The internode, *ca.* 2 cm long, was scored by shallow radial slits in the most distal end. The internode was allowed to incubate in an autoclaved buffer solution of 10 mM potassium phosphate and 3% sucrose until the epidermal tissue began to separate from the cortex at the score marks. The epidermis was peeled in strips with jeweler's forceps until the surface of the stem was completely peeled. To ensure complete removal of the epidermis from the entire stem segment, the strips were left attached to the end before

segmenting the internodes into 2 or 3 lengths of 0.5 cm each. Only one end of the internode was handled during the peeling process, and it was not used for any of the experimental treatments. Care was taken to make the cuts at right angles to produce the segments, and they were measured again to ensure accuracy of length. When an aliquot of the incubating medium was pipetted into the sterile petri dishes, sterile technique was used.

Auxin-Induced Growth Assay

In order to determine whether peeled and unpeeled segments of both wildtype and mutant varieties were capable of responding to auxin, the following experiment was undertaken. For each treatment, 10 segments were placed in 20 mL incubating solution in Parafilm-sealed petri dishes. To study the effect of auxin on stem segments, all experimental specimens were bathed in an autoclaved solution of 10 μ M α -naphthaleneacetic acid (NAA), following the procedure of Koch and Moore (1991), buffered in 10 mM potassium phosphate buffer and 3% sucrose at pH 6.5 (Schneider, 1938). Controls were incubated in an autoclaved solution of 10 mM phosphate buffer and 3% sucrose at pH 6.5. In order to determine whether or not the tissue elongation was indeed a response to auxin, tissue segments were placed in a solution of NAA and the H⁺-pump inhibitor, sodium orthovanadate. Sodium orthovanadate was used at a concentration of 200 mM (Cocucci *et al.*, 1980). Sterile technique was used during the transfer of solutions to eliminate fungal contamination. Incubations in all experiments were carried out for 5 days, and the length of the stem segments was measured with an ocular micrometer under a Wild stereomicroscope.

Kinetics

To further examine whether peeled and unpeeled mutant stem segments elongated at the same rate, we measured the change in length over time. Peeled and unpeeled stem segments of both garden pea varieties were bathed in an auxin solution and measured in length the first and fourth hour and then at 24 h intervals for the next 4 days. Similar time points over a 4-day period were employed for the buffer controls.

Transmission Electron Microscopy

To investigate the different responses to peeling between the *argenteum* mutant and the wildtype, the tissue was examined for a wound response by electron microscopy. Segments prepared and incubated for 48 h, as described above, were cut transversely into 2 mm long pieces and placed in cold, 3%, EM-grade glutaraldehyde containing 2% tannic acid in 0.05 M sodium cacodylate buffer at pH 6.8 for 2 h. The segments were then rinsed with agitation 3 times for 10 minutes each in cold, 0.05 M sodium cacodylate buffer, pH 6.8.

Postfixation was performed in cold, 2%, EM-grade osmium tetroxide (OsO₄) and 1% potassium ferricyanide in 0.05 M sodium cacodylate buffer, pH 6.8, for 2 h. The tissue was rinsed 5 times for 5 minutes each with cacodylate buffer and left at 4°C overnight. The tissue was pre-stained with cold, 2% aqueous uranyl acetate for 20 minutes and rinsed with cacodylate 3 times.

An acetone dehydration series was employed with 10, 25, 40, 50, 70, 85, 95, and three 100% incubations of 10 minutes each at room temperature. Two

additional dehydration steps were conducted with 100% propylene oxide for 10 minutes each. The tissue was then returned to 100% acetone (2 changes).

Infiltration was performed with increasing concentrations of Spurr's embedding medium (Spurr, 1969) in acetone, with each step at least 6 h long. The first infiltration step consisted of 6 parts acetone: 1 part Spurr's resin. The segments were then embedded in 100% Spurr's resin and cured for 8 h at 70°C.

Acid Phosphatase Reaction

To investigate the possibility that a wound reaction was occurring yet not detectable by secondary wall deposition, we administered the Gomori test to assay for acid phosphatase activity. In healthy plant cells, acid phosphatase is sequestered within the vacuole (Matile, 1975). When acid phosphatase activity is detected via the Gomori reaction in the cytoplasm, it is indicative of wounding (Moore and Walker, 1981) or autolysis of the cell (Gahan and McLean, 1969). In the presence of particular substrates, the Gomori reaction yields an electron-dense precipitate that is then detectable under the electron microscope. Additional stem segments, treated as described above and incubated for 48 h, were used to test for acid phosphatase activity, according to the method of Lewis (1992). Stem tissue from all treatments was first fixed in cold, 2%, EM-grade glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.8. Following the 2 h fixation, the segments were washed 3 times with cold cacodylate for 5 minutes each and 4 times with 0.05 M Tris-maleate buffer at pH 5.2 for 1 h. The segments were then incubated in a reaction mixture consisting of 40 mM Tris-maleate buffer, 8 mM sodium β -glycerophosphate (substrate), and 2.4 mM lead nitrate for 2 h at room temperature. Control

segments were incubated either in a solution without substrate or with substrate in the presence of a phosphatase inhibitor, 0.01 M sodium fluoride (Moore and Walker, 1981). The segments were then washed 5 times in cacodylate and post-fixed in 2% OsO₄ in cacodylate for 2 h. The tissue was dehydrated in a graded acetone series and infiltrated and embedded in Spurr's resin.

All blocks were sectioned with a Porter-Blum MT-2 ultramicrotome and post-stained with 2% aqueous uranyl acetate for 10 minutes and Sato's modified (Richard Giberson, Ted Pella, Inc., personal communication) lead stain for 5 minutes. Sections were viewed in a Zeiss EM 109 at 80 kV. Photographs were taken on Kodak Technical Pan 6415 120-mm film and developed in Kodak Dektol developer.

Light Microscopy

In addition to incubating stem segments in buffer or auxin as described above, living etiolated seedlings of both the wildtype and mutant variety were peeled *in situ* and left to grow exposed to air for 2 days. Segments from both varieties were also peeled and incubated in buffer solutions with and without auxin for 2 days. These segments were hand sectioned and stained with either 2% phloroglucinol in 13% HCl or with auramine O, a fluorochrome specific to lipoidal compounds, for 5 minutes and then viewed under bright field or epifluorescence optics, respectively, on a Zeiss research microscope.

Statistical Analyses

Preliminary analyses of the auxin-induced elongation data were done in order to set up the experimental design. Due to heteroscedasticity (unequal variances between treatments), the data were transformed by a log function, and the requirements for the Burr-Foster Q Test were met ($Q_{\text{calc}} = 0.1701$; $Q_{\text{crit}} = 0.1915$). Replicate sufficiency (3) was determined by the technique of Bros and Cowell (1987). Our sample size in the auxin-induced growth assay was 10. The Nested ANOVA (Zar, 1984) of the transformed data revealed that 10 subsamples were sufficient to accurately estimate growth ($p < 0.001$). As few as 5 subsamples would have been accurate ($p < 0.05$).

The auxin-induced growth experiments were done in order to determine whether stem segments lacking an epidermis were physiologically capable of plastic extension in response to auxin. A three-way factorial ANOVA (Zar, 1984) was used to test for interactions between three factors each with two levels: plant species (mutant or wildtype), treatment (peeled or unpeeled), and incubation medium (auxin or buffer). The mean for 10 subsamples was used for the 3-way factorial ANOVA. The mean was determined for the kinetics data using 10 subsamples and 5 replicates, as were 95% confidence limits (Zar, 1984).

RESULTS

Statistical Analyses

The three-way factorial ANOVA (Table 1) showed that there was a statistically significant ($p < 0.001$) interaction between the variety of plant, the presence or absence of epidermis, and the presence or absence of auxin. A significant interaction between factors indicates that no one factor alone is responsible for differences between treatments. The biological significance of the three-way interaction between factors is discussed below.

Auxin-Induced Growth Response

The progress of growth of the peeled and unpeeled *argenteum* mutant and wildtype varieties is shown in Fig. 2. With an intact epidermis, excised segments of the wildtype and mutant elongated rapidly (12.7% and 12.6%, respectively) for *ca.* 24 h in response to NAA. In contrast, peeled segments of the wildtype incubated in NAA grew little (2.3%) over the same time span. Peeled and unpeeled wildtype control segments (2.1% and 2.2%, respectively) and peeled and unpeeled mutant control segments (4.0% and 3.8%, respectively) in buffer alone also grew little in a 24 h period. Peeled segments of the *argenteum* variety elongated more (15.8%) than either the unpeeled wildtype (12.7%) or unpeeled mutant (12.6%).

In all treatments where tissue segments were placed in a solution with NAA and the H⁺-pump inhibitor, sodium orthovanadate, the segments failed to grow significantly (Fig. 3). Peeled and unpeeled mutant stem segments

elongated 3.0% and 3.5%, respectively. Peeled and unpeeled wildtype stem segments elongated 3.5% and 3.2%, respectively .

Growth Kinetics

When the change in length over time was measured, peeled and unpeeled mutant stem segments elongated at similar rates (Fig. 4). While the initial growth rates of the peeled and unpeeled mutant and unpeeled wildtype were similar (14%, 9%, and 13%, respectively), the peeled mutant was able to sustain this rate for a longer period of time, and hence these segments were routinely more elongated. The hormone-induced elongation was rapid for approximately 24 h in all of the treatments, after which the growth rate declined. All of the buffer controls (lacking auxin) showed a burst of elongation in the first 2 h but leveled off thereafter. Notably, the peeled wildtype segments grew more rapidly and to a slightly greater overall length than the buffered control segments. Some of the kinetic data recorded gave the impression that the segments were shrinking, as indicated on the graph as a downward slope. We believe that this was due to slight deviations from an exact transverse cut in generating the original segments followed by differential growth at the tip. With repeated measurements, the choice of ends was not consistent. Shrinkage of the segment tissue is highly unlikely.

Wound Response

The *argenteum* mutant and the wildtype tissue were examined for a wound response by electron microscopy. No evidence of suberin deposition was found in any of the peeled tissue segments that had been placed in aqueous

media, either auxin or buffer alone. Stem segments that were peeled and left exposed to the air (not submerged) showed a great deal of damage typical of mechanical wounding (Walker and Bruck, 1985). A necrotic layer several cell layers thick (Fig. 5a) and bearing collapsed cell walls (Fig. 5b) was observed by electron microscopy. Lamellar wall patterns were visible in the living cell layer immediately interior to the necrotic tissues lining the wound (Fig. 5c). This wall patterning resembled that of tissues that have become suberized (Bruck and Walker, 1985). The cell walls of peeled segments appeared normal, for the most part, although there were areas where the cell contents were disorganized and cell walls were torn (Fig. 5d). Suberization and lignification were monitored in peeled segments by histochemical methods. When peeled wildtype tissue was exposed to air for two days and stained with phloroglucinol, a lignin-specific stain, there was no indication of lignification in the peeled area. Additionally, there was no binding of auramine O, a fluorochrome used to identify suberin and other lipoidal substances.

Fig. 6a reveals the polylamellate appearance of the primary cell walls of the *argenteum* mutant. No secondary wall material, either lignin or suberin, was deposited in the cell wall of the peeled mutant (Fig. 6b). Similarly, suberin deposition was not detectable in the peeled wildtype segments incubated in auxin (Fig. 6c) or in buffer solutions (Fig. 6d). Furthermore, neither peeled wildtype nor peeled mutant segments incubated in buffer stained with either phloroglucinol or auramine O. However aggregations of Golgi vesicles in proximity to the plasma membrane were observed frequently in the living cell layer interior to the peeled area of the wildtype stem (Fig. 6c). Vesicle

aggregations outside the plasma membrane in this cell layer, presumably resulting from exocytosis, were also common in the peeled wildtype (Fig. 6d).

Acid Phosphatase Activity

The Gomori reaction creates an electron-dense precipitate in the presence of acid phosphatase that is prominent when viewed with an electron microscope. Acid phosphatase activity was observed in the cell walls of segments of both the peeled mutant (Fig. 7a) and the unpeeled wildtype varieties (Fig. 7b, c). Acid phosphatase activity was seen neither in the ER (Fig. 7b) nor in the Golgi apparatus (Fig. 7c) of the peeled mutant (Fig. 7a) or unpeeled wildtype segments. However, it was localized in the cytosol (Fig. 7d, 8a, b), intercellular spaces (Fig. 7d), ER lumina (7d, 8a), and mitochondria (7d, 8a, b, d) and was associated with other intracellular membrane systems (Fig. 8c, d) of the peeled wildtype. The fixation of this material was not as complete as would ideally be needed for revealing precise locations of the precipitate in the Gomori reaction. The reaction needs to be optimized for incubation time and substrate concentration, and fixation must be improved to better preserve cellular ultrastructure.

DISCUSSION

Auxin-Induced Growth

An absence of growth in peeled stem segments and coleoptiles has been reported by numerous investigators (Thimann and Schneider, 1938; Penny, 1969; Tanimoto and Masuda, 1971; Masuda and Yamamoto, 1972; Penny *et al.*, 1972; Yamagata *et al.*, 1974; Yamamoto *et al.*, 1974a, 1974b; Brummell and Hall, 1980; Pope, 1982; Kutchera *et al.*, 1987). Their interpretation of this result was

that the epidermis is the only auxin responsive tissue of the organ. Contradictory results have been obtained by other researchers. Thimann and Schneider (1938), Rayle (1973), Cleland (1975), and Peters *et al.* (1992) reported marked auxin-induced elongation in peeled oat coleoptiles, indicating that other cell layers are auxin responsive. Cleland (1991) observed that peeled oat coleoptiles elongate at the same rate and amount as unpeeled segments in the presence of exogenous auxin. Curvature due to growth occurred in as many peeled oat segments placed in auxin solutions as in unpeeled segments (Cleland, 1991). The epidermis is also unnecessary for growth regulation and gravitropic responses in maize roots (Cleland, 1991). Recently, growth has also been observed in peeled pea stem segments (Rayle *et al.*, 1991).

The chief rationale for this contribution was to attempt to reconcile these two contradictory bodies of evidence, one showing growth after epidermal peeling and the other its absence. We hypothesized that the lack of growth was due to a wound response in the tissues underlying the peel. That response would then interfere with cell-expansion machinery to block elongation of the organ. Growth of peeled organs would then be attributed to little more than luck and technical acuity, as a cleanness of peeling must have been effected to avert the wound reaction. The vagaries of chance were avoided in our study by the use of the *argenteum* mutant of the garden pea. We have accumulated evidence from scanning EM and other means that a wound response does not ensue from the peeling of this variety (data not shown, in preparation). Thus this variety stood as an ideal specimen for consistently and definitively testing the ability of peeled stems to grow and for testing the wound-response hypothesis to explain growth inhibition.

In this research, peeled segments of the wildtype pea responded minimally to 10mM phosphate-buffered (pH 6.5) auxin, although to a greater extent than wildtype segments in buffer alone. Therefore, auxin-induced growth, although limited, was observed even in the nonmutant organ devoid of an epidermis. Growth, however, was much less than in the pea stem segments used by Rayle *et al.* (1991). This kind of difference was explained by other researchers' use of higher strength buffers (more than 5 mM), preincubation in acidic distilled water or buffer, or tissue damage (Rayle *et al.*, 1991; Rayle and Cleland, 1992). Their acidic distilled water explanation for reduced growth of abraded or peeled segments is that the acid-induced growth stimulated by the acid environment somehow deterred subsequent auxin-induced growth, preventing appreciable segment elongation overall. This would not account for the minimal growth of our nonmutant segments, as they were buffered at pH 6.5; in addition, our distilled water had an alkaline pH (greater than 8.5).

More notably, stem segments of the *argenteum* mutant, from which the epidermis had been removed, responded well to auxin, elongating in response to auxin more than either the unpeeled wildtype or unpeeled mutant segments. Because auxin-induced growth occurred in the mutant in the absence of the epidermis, it is apparent that the epidermis is not required for auxin-mediated growth and must not be the sole target tissue for auxin. The assertion that segment growth is inhibited by high molarity buffers, as made by Rayle *et al.* (1991), is challenged by these results, as both peeled and intact (unabraded and unpeeled) segments in our investigation grew in the presence of a "high" (10 mM) buffer. Of the hypotheses offered by Rayle *et al.* (1991) and Rayle and Cleland (1992) for growth inhibition of peeled segments, only the

one regarding tissue damage during the peeling process appears reasonable in light of our results. Our supposition is that tissue damage does occur with peeling (*cf.* Kutchera *et al.*, 1987), so that the other hypotheses are unnecessary to explain growth inhibition.

The controversy over the role of the epidermis is in need of resolution due to the emerging evidence concerning auxin receptors. The notion that the epidermis is the exclusive auxin-responsive tissue is being used as evidence in support of epidermal restriction of auxin receptors. Because oat and maize coleoptiles (Cleland, 1991), lupine hypocotyls (Penny *et al.*, 1972), and epicotyls of the wildtype pea (Rayle *et al.*, 1991, and this report), and the *argenteum* mutant of the pea in this report are capable of auxin-induced elongation without an intact epidermis, this receptor model cannot be valid. The auxin-binding protein (ABP) identified in the epidermis by immunocytochemistry by Löbner and Klämbt (1985b) may be a true auxin receptor, but there must be others in underlying tissues to account for this apparent discrepancy. Löbner and Klämbt (1985b) conceded that the auxin receptor may be localized in internal tissues, just not discernible by their assay.

Additionally, antibodies raised against the auxin-binding domain of ABP1 from maize inhibit growth of both excised and longitudinally split coleoptiles (Löbner and Klämbt, 1985a), arguing for the designation of ABP1 as a true receptor. These antibodies further impede auxin-induced hyperpolarization of tobacco mesophyll protoplasts, an event presumably mediated by the plasma membrane-bound H⁺-ATPase that acts to acidify the cell wall during auxin-induced cell expansion (Barbier-Brygoo *et al.*, 1989). Incubation with antibodies raised against a plasmalemma ATPase from yeast also suppress the

transmembrane potential difference. Hyperpolarization and proton excretion have been reported to occur in auxin-incubated, peeled segments (Peters *et al*, 1992). These results demonstrate the localization of an ABP in nonepidermal tissue.

Wound Response: Cell-Wall Deposition

In an attempt to explain the discrepancy between the auxin responses of the mutant and wildtype segments, tissues were examined for wound effects. Oat coleoptile segments peeled to different degrees or lacerated by deep longitudinal slits by Pope (1982) resulted in growth inhibition proportional to the extent of the damage. However, peeling was less inhibitory than lacerating, suggesting the former causes a wound response too limited to account for the comprehensive growth suppression of peeled segments. Brummell and Hall (1980) generated clean peels, although consisting of two cell layers, that separated at the tangential walls of the underlying cells. Thus the cortical cells were not ruptured, and the authors maintained that little damage occurred. However, their electron micrograph showed cytoplasmic disruption in the external cells of the segments, indicative of a wound response, and their segments failed to elongate. On the other hand, Kutchera *et al.* (1987) found that the epidermal cells were significantly damaged by peeling, as they were unreactive with the vital dye, neutral red, and they failed to osmoregulate in hypertonic solutions. If epidermal cells were wounded by this process, it is likely that adjacent internal cells would also be wounded.

We discovered little indication of progressive secondary wall deposition in peeled wildtype pea stems during the hours following auxin application,

when auxin-induced growth would be expected. When bathed by aqueous solutions corresponding to those used in the growth experiments, peeled segments were devoid of observable deposition of secondary wall materials, as determined by histochemistry with lignin-specific (phloroglucinol) and suberin-highlighting (auramine O) stains as well as by EM. Thus suberin and lignin may not be laid down in mechanically wounded tissue incubated in an aqueous medium. Suberin deposition was absent in wounded hardwood tissue tree segments kept under water for several weeks (Schmitt and Liese, 1993). Only when unsegmented wildtype pea stems were peeled *in situ* and exposed to air in our study did lamellar wall patterns become visible in the living cells underlying the peeled surface. If desiccation is not imminent, the plant may refrain from laying down unnecessary protective layers.

Suberin and lignin are biologically expensive polymers for an etiolated, annual pea seedling. Other wound responses might be more feasible. Pre-existing (hydroxy)proline-rich cell-wall proteins (HRGPs) become insoluble due to H₂O₂-mediated oxidative crosslinking (Bradley *et al.*, 1992). These HRGPs have been found to proliferate in response to wounding in taxonomically diverse species (Cardemil and Riquelme, 1991). This extensive crosslinking has been observed in response to fungal elicitors and mechanical wounding (Bradley *et al.*, 1992). It was assumed to create a barrier to the impending infection, yet at the same time it made the walls and tissue more rigid; therefore, the capacity for cell expansion would be diminished. The differential responses of peeled and unpeeled tissue segments may be explained by this phenomenon. Because this crosslinking is complete within 10 minutes (Bradley *et al.*, 1992), it would prohibit auxin-generated growth,

which begins within 15 minutes after incubation in our segments and in those examined by others (Penny, 1969; Penny *et al.*, 1972, Yang *et al.*, 1993).

The possibility still remains, nonetheless, that deposition of elongation-impeding wall compounds was underway but had not advanced to a prominent stage in the wall itself. In many peeled wildtype segments examined under the EM, there was extensive vesiculation by the Golgi apparatus and accumulation of exocytotic vesicles on both sides of the plasma membrane of the living, intact cells immediately subjacent to the peeled area. Fusion of Golgi vesicles with the plasma membrane would be expected were wall materials, such as suberin and lignin, being actively deposited. If the peeled wildtype tissue were allowed to incubate for a longer period of time, this apparent onset of secondary wall formation might be extended and manifested in the distinctive lamellar configuration of suberized walls, substantiating extensive wound repair. Schmitt and Liese (1993) observed suberin deposition in many species of hardwoods only after three or more weeks following wounding.

This pronounced Golgi activity and putative exocytosis were absent in unpeeled (control) wildtype stems and in peeled mutant stems. If these events are attendant on a wound reaction, then their absence in peeled mutant segments suggest an avoidance of wound recognition. The stem segment elongation of the mutant in response to auxin, as reported here, would be explainable by the alleviation of the elongation-obstructing wound response.

The acid phosphatase procedure yielded intriguing results despite it being a single attempt with a standardized protocol that demands optimization for the species investigated. Acid phosphatase activity was restricted to the cell walls

in both unpeeled wildtype and peeled mutant varieties of the pea, indicating that autolytic processes were not prevalent. This localization is typical of healthy, non-senescing cells (Matile, 1975; Moore and Walker, 1981). The hydrolase activity within the cytosol, organelles, and intercellular spaces of the cells underlying peeled regions of wildtype stem segments was indicative of the occurrence of autolysis as a wound response leading to cell death. Active expansion of these cells would presumably be deterred. Furthermore, cell-to-cell signaling of the wound response might preclude adjacent cells from responding to auxin and thereby hindering elongation of the organ as a whole. The level of acid phosphatase activity in our segments, incubated at 25°C, was not representative of most wound responses. A greater abundance of precipitate is usually observed around membranes and organelles when the Gomori reaction is incubated at 37°C.

Given the rejection of the hypothesis that the epidermis is the exclusive site of action of auxin in the growth of stem segments, a wound response remains the most promising explanation for the disparity in growth between peeled and unpeeled segments of nonmutant plants. While the wound reaction may not be initially manifested by wall building activities, other wound reactions could inhibit the mesophyll tissue from responding to auxin. Future research in our laboratory will include optimization of the acid phosphatase assay to resolve the issue of a wound effect in the peeled stem segments.

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Figure 1. Schematic diagram of G-protein signal-transduction cascade.

Figure 2. Pea stem elongation in the presence and absence of an epidermis.

Figure 3. Pea stem segment elongation in the presence of the H⁺-pump inhibitor, sodium orthovanadate. *Arg*, *argenteum* mutant; *WT*, wildtype; +*A*, auxin present; -*A*, auxin absent; +*P*, peeled segment; -*P*, unpeeled segment.

Figure 4. Rate of auxin-induced growth of mutant and wildtype stem segments. *Arg*, *argenteum* mutant; *WT*, wildtype; +*A*, auxin present; -*A*, auxin absent; +*P*, peeled segment; -*P*, unpeeled segment.

Figure 5. Electron micrographs of wildtype stem segments peeled and left exposed to air. a. Collapsed cells on periphery of stem segment beneath peel. x15,600. Bar, 2 μm . b. Suberin-like substance (arrow) deposited in wall (CW) of a cell underlying the peel. x53,400. Bar, 0.2 μm . c. Collapsed cells (CC) on periphery of stem segment beneath peel. CV, central vacuole. x35,600. Bar, 0.2 μm . d. Tissue underlying the peel appeared torn and disorganized. x4,400. Bar, 2 μm .

Figure 6. Electron micrographs of both mutant and wildtype stem segments peeled and incubated in auxin or buffer. a. Peeled mutant stem segments incubated in buffer showing no evidence of secondary wall deposition. x36,400. Bar, 0.5 μ m. b. Peeled mutant segments incubated in auxin showing no suberin or lignin deposition. *E*, etioplasts; *M*, mitochondrion. x23,500. Bar, 0.5 μ m. c. Secondary wall deposition was absent in peeled wildtype segments incubated in auxin, although vesicle proliferation from the Golgi apparatus (*G*) was common, and multivesicular bodies (*V*) occurred alongside cell wall near the peel. x60,000. Bar, 0.2 μ m. d. Visible secondary wall deposition was absent in wildtype stem segments incubated in buffer. Large groupings of vesicles (*V*) were frequent near walls in cells adjacent to peeled areas. *ER*, endoplasmic reticulum. x91,000. Bar, 0.5 μ m.

Figure 7. Electron micrographs of acid phosphatase activity in peeled and unpeeled stem segments of mutant and wildtype peas. Bar, 0.2 μm . a. Low level acid phosphatase activity was present only in the cell wall (CW) of the unpeeled mutant, as indicated by unlabeled arrow. Both the Golgi apparatus (G) and cytoplasm were free of lead precipitate in peeled mutant segments. $\times 152,000$. b. Unpeeled wildtype segments showing acid phosphatase localization in the cell wall but not in the endoplasmic reticulum (ER) or cytoplasm. $\times 95,000$. c. Acid phosphatase activity absent from the Golgi apparatus and cytoplasm of unpeeled wildtype stem segments. Hydrolase activity present in the cell wall near the peeled area, as indicated by unlabeled arrow. $\times 150,000$. d. Peeled wildtype stem segments showing acid phosphatase activity in the cytoplasm, endoplasmic reticulum (ER), other organelles (at leftmost arrow, probably a mitochondrion), and intercellular spaces (at arrow at top-right) near the peeled area. Acid phosphatase activity absent from the vacuole (CV) and cell walls (CW) of this tissue. $\times 110,000$.

Figure 8. Electron micrographs of acid phosphatase activity in peeled wildtype pea stem segments. Bar, 0.2 μm . a. Low level hydrolase activity in the endoplasmic reticulum (at arrow in cell on right, despite lack of resolution of limiting membrane), other organelles (at arrow in cell on left, probably a mitochondrion), and the cytosol of peeled wildtype stem segments. Activity absent from central vacuole (CV). x66,600. b. Acid phosphatase activity localized in mitochondria (at leftmost arrow) and cytosol (rightmost arrow). Activity absent in central vacuole (CV). x55,000. c. Enzymatic activity distinctly localized around membrane of a plastid, possibly an etioplast or amyloplast (at arrow and elsewhere). It was also present in the surrounding cytosol. x60,000. d. Ubiquitous acid phosphatase activity within organelles, possibly etioplasts or mitochondria (at arrows), and in the cytosol of peeled wildtype segments. x75,000.

Table 1. Comparison of means to evaluate whether the epidermis is necessary for auxin-induced growth in wildtype and mutant varieties of the garden pea. The dependent variable is the log-transformed value for change in length (mm). Three factors with two levels each are plant (mutant and wildtype), peel (peeled and unpeeled), and auxin (present and absent). The three-way interaction is significant ($p < 0.001$).

ANALYSIS OF VARIANCE

<u>SOURCE</u>	<u>SUM-OF-SQUARES</u>	<u>DF</u>	<u>MEAN-SQUARE</u>	<u>F-RATIO</u>	<u>P</u>
PLANT	1.543	1	1.543	21.045	0.000
PEEL	3.499	1	3.499	47.732	0.000
AUXIN	0.299	1	0.299	4.084	0.047
PLANT* PEEL	0.587	1	0.587	8.000	0.006
PLANT* AUXIN	0.180	1	0.180	2.457	0.121
PEEL* AUXIN	0.069	1	0.069	0.938	0.336
PLANT* PEEL* AUXIN	1.416	1	1.416	19.316	0.000
ERROR	5.279	72	0.073		