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HORMONE-INDUCED SIGNALING DURING MOSS DEVELOPMENT

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ABSTRACT

Understanding how a cell responds to hormonal signals with a new program of cellular differentiation and organization is an important focus of research in developmental biology. In *Funaria hygrometrica* and *Physcomitrella patens*, two related species of moss, cytokinin induces the development of a bud during the transition from filamentous to meristematic growth. Within hours of cytokinin perception, a single-celled initial responds with changes in patterns of cell expansion, elongation, and division to begin the process of bud assembly. Bud assembly in moss provides an excellent model for the study of hormone-induced organogenesis because it is a relatively simple, well-defined process. Since buds form in a nonrandom pattern on cells that are not embedded in other tissues, it is possible to predict which cells will respond and where the ensuing changes will take place. In addition, bud assembly is amenable to biochemical, cellular, and molecular biological analyses. This review examines our current understanding of cytokinin-induced bud assembly and the potential underlying mechanisms, reviews the state of genetic analyses in moss, and sets goals for future research with this organism.

CONTENTS

INTRODUCTION 502
MOSS GAMETOPHYTE DEVELOPMENT 502
CELLULAR CHANGES DURING BUD ASSEMBLY 505
SUBCELLULAR CHANGES UNDERLYING BUD ASSEMBLY 505
SIGNALS INITIATING BUD ASSEMBLY 508
Light 508
Hormones 509

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CALCIUM AS AN INTRACELLULAR MESSENGER IN BUD ASSEMBLY 511
PROSPECTS FOR THE ANALYSIS OF BUD ASSEMBLY 512
Early Events 512
Later Events 514
GENETIC ANALYSES OF BUD ASSEMBLY 515
IMMEDIATE GOALS FOR MOSS RESEARCH 517
Generation of Additional Developmental Mutants 519
CONCLUDING REMARKS 520

INTRODUCTION

The study of nonflowering plants has contributed important information about the nature of changes in form and function that occur during the development of both flowering and nonflowering plants. While even these simple plants grow and develop via complex processes and interactions, their less complicated morphology makes the study of certain aspects of development more feasible than is possible in higher plants. In *Funaria hygrometrica* and *Physcomitrella patens*, two related species of moss, assembly of a bud from an initial cell involves hormone-induced organogenesis beginning in a single cell that is not embedded in other tissues. In this review, we describe what is known about early events in bud assembly, examine the advantages and limitations of studying moss to understand the underlying elements of eukaryotic development, and identify areas of future investigation and the tools that will be critical for these studies. Finally, we set some immediate goals for the study of development in moss.

MOSS GAMETOPHYTE DEVELOPMENT

The earliest stage of vegetative development in *Funaria* and *Physcomitrella* is characterized by cellular differentiation during filament growth. The cellular dimensions and timing of the events described here for *Funaria* grown in culture have been recently described in detail (56). Spore germination (Figure 1*A* and *B*) leads to the formation of a filament that consists of a tip (apical) cell and a linear array of subapical cells produced by successive divisions of the tip cell. These cells, the chloronema (Figure 1*C*), are filled with disc-shaped chloroplasts and have cross walls that are perpendicular to the filament axis. As is characteristic of the subapical cells of tip-growing organisms (36), no further growth occurs in the subapical chloronema cells. The tip cell elongates, reaches a maximum length, and divides to produce a new subapical cell to extend the filament. Chloronema filament growth continues until, in response to increases in light (18) and auxin, the appearance of the chloronema tip cell begins to change. This cellular differentiation leads to formation of the second filament

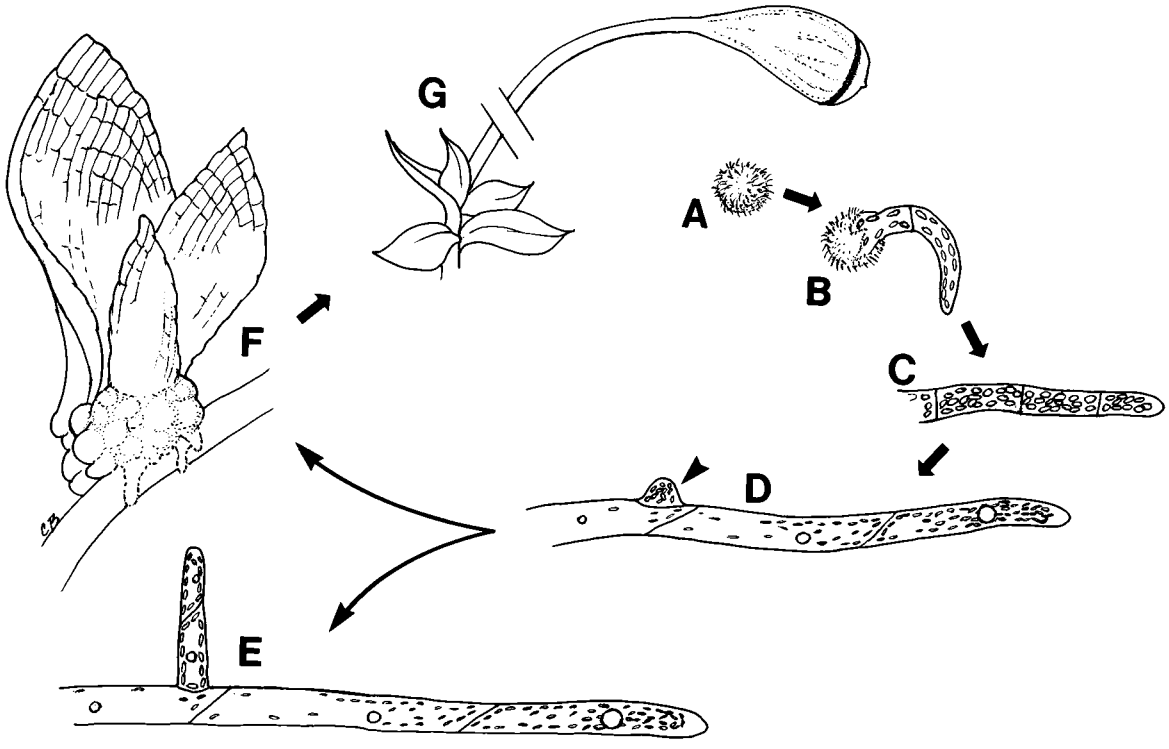

Figure 1 Stages of moss development. Haploid spores (*A*) germinate to form a filament consisting of chloronema cells (*B* and *C*). Subsequently, light and auxin induce changes in the tip cell to give rise to caulonema cells (*D*). A single-celled initial (*D, arrowhead*) forms on the second subapical cell of the caulonema filament. This initial cell has two potential fates. In the absence of cytokinin, the initial cell will continue to grow by tip growth to form a new lateral filament (*E*). In the presence of cytokinin, the initial cell takes on the morphology associated with the assembly of a bud to form the leafy shoot (*F* and *G*) that eventually bears the gametangia (not shown). Following fertilization, a diploid capsule (*G*) forms on the leafy shoot. Ultimately, meiosis occurs within the capsule to produce haploid spores.

cell type, the caulonema (Figure 1*D*). In comparison to a chloronema tip cell, a fully developed caulonema tip cell elongates dramatically, exhibits decreased time for tip cell division, and has smaller, elongated, flattened chloroplasts that contain less chlorophyll. During the transition from chloronema to caulonema, the newly formed cells appear intermediate in character between the two cell types, but after five to six days, caulonema cells are long, nearly clear, and have cross walls that are oblique to the filament axis.

Once caulonema cell differentiation has begun, a new axis of cellular polarity is set up during the formation of initial cells. Very shortly after division of a caulonema tip cell, a small swelling appears in the second subapical cell (the third cell of the filament). This outgrowth, which will give rise to an initial cell (Figure 1*D*, *arrowhead*), appears at the apical end of the cell near the apical-most end of the oblique cross wall. The outgrowth continues to expand, and the division that will produce the fully formed initial cell occurs five to six hours after visible evidence of initial cell formation is first seen. Before this division, the nucleus migrates from midway in the filament cell to the initial cell site, where it divides. One daughter nucleus moves into the forming initial cell, and the second moves back to the middle of the filament cell. A cell wall, oriented parallel to the longitudinal axis of the filament cell, separates the initial cell from the filament to produce the fully formed initial cell (Figure 1*D*).

The next stages of development in *Funaria* are characterized by hormone-induced organogenesis as a bud is assembled from the caulonema initial cell and the leafy gametophyte develops from the bud. The caulonema initial cell has two potential fates that are developmentally distinct. In the absence of cytokinin, the initial cell will continue to grow by tip growth to produce a new lateral filament (side branch) (Figure 1*E*), thus maintaining the filamentous growth habit. However, in the presence of cytokinin, the initial cell takes on a distinct morphology associated with the assembly of a bud in transition from filamentous to meristematic growth.

In culture, bud assembly can occur in both the presence and absence of exogenous hormone; however, treatment with cytokinin leads to the production of significantly more buds. Tissue can respond to added cytokinin for a period of time after caulonema initial cells begin to form, but prior to the appearance of buds in untreated tissue (8, 13). The very small number of buds that form in the absence of added cytokinin presumably arise in response to endogenous hormone.

Early changes during bud assembly include an altered pattern of cell expansion and elongation of the initial cell to produce the single-celled bud. Later changes involve divisions within the bud to give rise to a simple meristem that produces a leafy shoot (Figure 1*F* and *G*) that eventually bears the gametangia (not shown). Following the production of gametes and fertilization, the zygote

develops into a sporophyte (Figure 1*G*) with a stalk 3–4 cm long that will bear a single capsule containing hundreds of thousands of haploid spores.

In the context of this description of moss development, we turn our discussion to what is known at the cellular and subcellular levels about how the caulonema initial cell is assembled into a bud.

CELLULAR CHANGES DURING BUD ASSEMBLY

Cellular changes are apparent in the initial cell within two to three hours after the addition of cytokinin. At the time of the division that separates the initial cell from the filament cell, the initial cell (Figure 2*A*) is approximately 20 μm long and contains many large chloroplasts. The first visible indication of cytokinin-induced bud assembly is the dramatic swelling of the initial cell resulting from a lack of further tip growth and a change in the pattern of cell expansion and elongation. The apical area of the initial cell becomes dome-shaped as the deposition of new wall material moves from the very tip region to the sides of the cell, forming a rounded single-celled bud with an elongating stalk (Figure 2*B*) (10, 16). Other early cellular changes in the single-celled bud include a reduction in chloroplast size and an alteration in chloroplast shape. The first division of the bud occurs when it is approximately 65 μm long. This division is asymmetric and, therefore, produces daughter cells of different developmental fates. The bud divides transversely with respect to its long axis, producing a large, highly vacuolate stalk cell and a small, densely cytoplasmic apical cell (Figure 2*C*). The apical cell then divides longitudinally, resulting in two densely cytoplasmic cells (Figure 2*D*) (16). Subsequent unequal cell divisions give rise to a tetrahedral apical cell (meristem) that continues to divide in three planes to form the relatively simple multicellular bud (Figure 2*E*). The subapical cells of this bud then divide more frequently than the apical cell to give rise to a larger, more complex bud (Figure 2*F*) (24). Subsequently, leaf primordia arise as projections from the side of the bud (Figure 2*F*, *arrowhead*). One of the derivatives of each apical cell division gives rise to one primordium; the leaflets (Figure 2*G*) derived from it are composed of files of cells that grow by general expansion.

SUBCELLULAR CHANGES UNDERLYING BUD ASSEMBLY

During initial cell formation there is a change in the cellular organization at the presumptive initial cell site; directed growth at this site takes place via stratification of organelles (16). TEM (transmission electron microscopy) studies have shown that the apex of the outgrowth contains Golgi bodies, associated

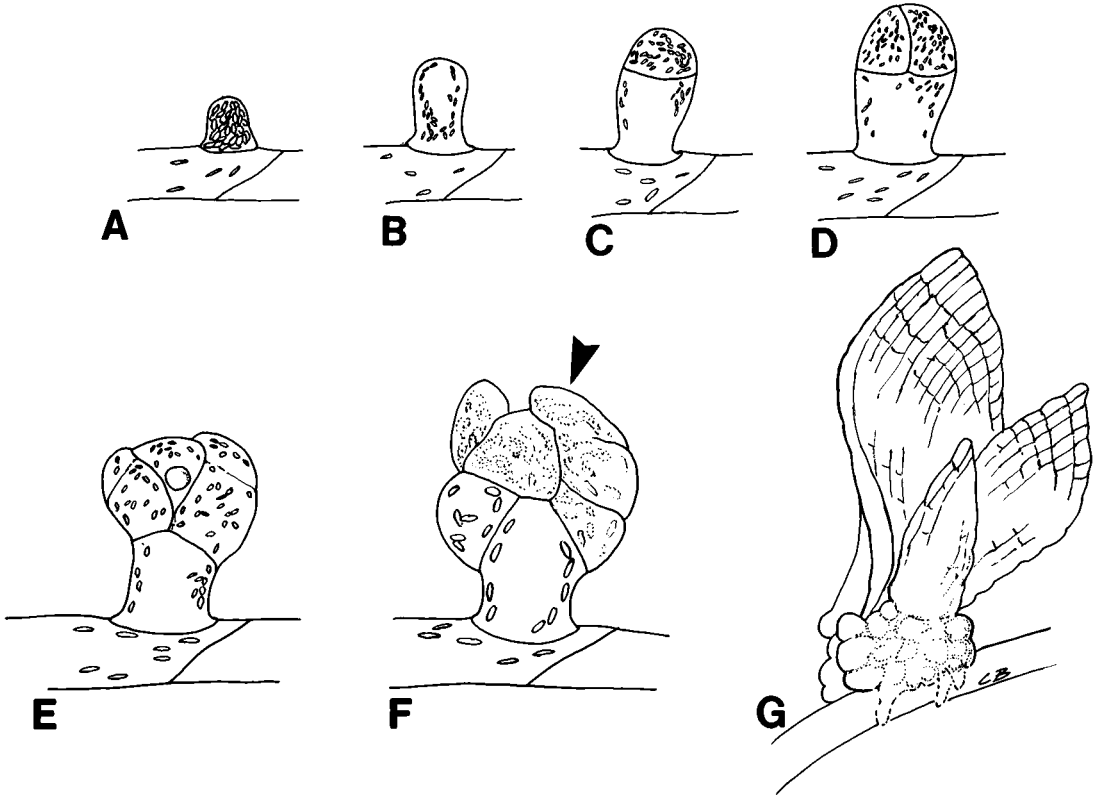

Figure 2 Developmental transition from filamentous to meristematic growth. Changes are apparent in the initial cell (*A*) within two to three hours of cytokinin addition. The first visible indication of bud assembly is a dramatic swelling of the initial cell (compare *A* and *B*). This is followed by an asymmetric division to produce a large, highly vacuolate stalk cell and a small, densely cytoplasmic apical cell (*C*). The apical cell divides longitudinally, resulting in two densely cytoplasmic cells (*D*). Subsequent unequal divisions give rise to a tetrahedral apical cell that continues to divide in three planes to form the relatively simple multicellular bud (*E*). The subapical cells of the bud divide more frequently than the apical cell to give rise to a larger, more complex bud (*F*). Subsequently, the leaf primordia (*F*, *arrowhead*), each of which will develop into a leaflet of the leafy shoot (*G*), arise as projections from the side of the bud.

vesicles, and cortical (immediately adjacent to the plasma membrane) endoplasmic reticulum (ER). The outgrowth is filled with cytoplasm but contains only a few vacuoles and chloroplasts that are positioned in the cell cortex. While tip-growing cells are distinguished by perpetual stratification of organelles (35), the moss initial cell loses this organization after the division separating it from the filament occurs (16).

A fully formed initial cell that has not been stimulated to form a bud becomes a side branch, and the cellular organization resumes the tip cell pattern of organellar stratification. If perception of cytokinin occurs, organelle distribution remains random during the dramatic swelling that follows, but there is a qualitative and quantitative change in internal membranes (41, 42). The structure, quantity, and distribution of the ER during bud assembly have been studied using both the fluorescent, lipophilic carbocyanine dye, 3,3′-dihexyloxacarbocyanine iodide, and rapid freeze-fixation/freeze-substitution. These studies have shown that while the cortex of the bud contains the same cellular components as side branches, during bud assembly there is an increase in ER membrane density and cortical ER volume. The ER network becomes "tighter" and forms a gradient within the developing single-celled bud as the stalk region becomes delineated. As vacuolation increases in the stalk region, its ER network becomes more open. In contrast, the apex of the one- or two-celled bud has closely packed ER. This ER is associated with ribosomes and forms a shell in the periphery of the bud apex with close apposition of the outermost ER and plasma membrane throughout the bud cortex. The new configuration and quantity of ER has been found to be the most significant subcellular change observed during bud development, and this bud-like pattern has never been observed in side branches. The ER continues to be closely spaced in the apical region of the bud as it develops into a multicellular structure and forms the tetrahedral apical cell. It is not clear how the change in cortical ER density during bud assembly is accomplished.

McCauley & Hepler (42) suggested that the cortical ER may be a general indicator of the metabolic status of a cell during bud assembly. Cytokinin-induced bud assembly may be mediated through release of calcium, and the increased quantity of cortical ER in buds may represent the mechanism to change calcium sequestration capabilities and intracellular calcium levels.

Doonan et al (24) demonstrated that reorganization of the microtubule cytoskeleton is also correlated with changes in the pattern of cell growth during bud assembly. In a newly formed caulonema initial cell, there is a meshwork of microtubules that have a random orientation and do not focus to any particular site in the cell. As an initial cell that has not been stimulated to form a bud resumes tip growth, the microtubules associated with the nucleus become aligned along the axis of cell elongation and focus to the surface of the tip apex. In

cytokinin-treated initial cells, the nucleus-to-cortex microtubules are oriented randomly and do not focus to the tip, which is consistent with the more diffuse pattern of growth in the bud. Evidence from this study suggests that cytokinin may be specifically affecting the cytoplasmic microtubules in the developing bud. While cytokinin has no apparent effect on either microtubules in nonbud-forming tissue or on spindle and phragmoplast microtubules within the bud, the cytoplasmic microtubules in assembling buds appear to be poorly preserved. Based on this lack of microtubule preservation, it has been suggested that the cytokinin-induced changes in cytoplasmic microtubule organization may account for the loss or prevention of tip-directed growth resulting in a swelled initial cell.

SIGNALS INITIATING BUD ASSEMBLY

Experimental evidence supports the existence of two distinct developmental stages in bud formation: (*a*) caulonema initial cell formation and (*b*) assembly of the bud from the initial cell (11). In many of the studies outlined below, these two processes have not been distinguished from one another. Where possible, we have tried to separate them and to focus our discussion on bud assembly.

Light

Light has a marked influence on a number of processes in moss development such as spore germination, growth of chloronemal and caulonemal side branches, and bud assembly (3, 4, 18, 19, 60). Evidence suggesting a light requirement in bud formation includes the absence of buds in dark-grown plants and the induction of buds in dark-grown plants exposed to light (3, 4, 19, 60). Because the starting tissue for these studies was not defined in most cases, we cannot conclude whether initial cell formation, bud assembly, or both are light-sensitive.

Some of the characteristics of the light requirement for bud formation have been determined. Production of buds is dependent on the intensity of red light, and weak white light delays further development to the leafy gametophyte (3, 44, 60). In low-intensity continuous light, buds do not assemble from initial cells in response to cytokinin (18). However, as white light intensities are raised, bud formation increases steadily (3). Large numbers of buds can also be induced with exposure of tissue to red light with maximum bud production occurring at >16 μmol quanta $m^{-2} \cdot s^{-1}$ (3). It has been shown in *Physcomitrium turbinatum* that there is a relatively large cumulative light dose required for bud formation, suggesting that light energy may be used for the synthesis of a product that must accumulate before buds form (44). Further support for the accumulation of such a product comes from experiments in which moss tissue "remembers" exposure to light. In *Physcomitrella*, some buds are

formed when dark-grown cultures are exposed to light for several hours and then simultaneously treated with cytokinin and returned to darkness (18).

Hormones

In assessing the role of regulatory molecules in caulonema filament and initial cell formation, we have previously suggested criteria that would need to be satisfied to show the involvement of a molecule in a physiological process (56). For example, this effector should be present at the appropriate times and in the correct concentrations to elicit the response. This requires mechanisms to alter the amounts of the effector or the sensitivity of the target cell to the effector. It should also be possible to show that altering the level of the effector in wild-type or mutant plants changes the response. We will use these criteria to provide a framework with which to evaluate the evidence implicating auxin and cytokinin in bud assembly.

The genetic nomenclature that follows uses the respective authors' strain designations. Italicized lower-case letters represent mutant alleles that have been shown in crosses to segregate in a Mendelian manner. Italicized upper-case letters indicate strain designations based on phenotypic analyses; it has not been determined that these strains are the result of single mutations (5, 26).

AUXIN To our knowledge, auxin levels have not been measured during bud formation. However, evidence of a role for auxin in bud formation comes from experiments with cytokinin-resistant (*b*enzyl*a*denine-*r*esistant, *BAR*) mutants of *Physcomitrella*. *BAR* mutants do not produce buds even in the presence of exogenous cytokinin (3, 5). When grown in white light on medium lacking hormones, one class of *BAR* mutants produces a normal amount of caulonema but forms few or no leafy gametophytes even though these mutants are producing initial cells. With the addition of low levels of auxin, however, these mutants show normal development through the formation of leafy gametophytes. These results suggest that in addition to cytokinin, bud assembly requires auxin, but presumably at higher levels than is needed to produce caulonema cells (3, 56).

CYTOKININ At the time of the last comprehensive review in this series on gametophyte development in ferns and bryophytes (12), it had been shown that addition of cytokinin to moss cultures stimulated bud formation (see 13 and references therein). Since that time, it has been shown that cytokinin specifically affects both initial cell formation and the subsequent assembly of a bud. Bopp & Jacob (11) have shown that in *Funaria* picomolar levels of cytokinin induce a caulonema filament to produce initial cells, whereas nanomolar to micromolar concentrations are required for the assembly of a bud from an initial cell.

All synthetic and natural substances with the characteristics of a cytokinin (adenine derivatives with an N^6-substituted side chain of five or more carbon atoms) evaluated to date can cause a change from filamentous growth to at least

the early stages of bud formation (9). Both cytokinin bases and ribosides are active; however, the ribosides are less so (67). Additional studies have shown the concentration dependence of cytokinin action over a range of 50 nM to 1 μM (13, 67).

Few measurements of tissue-derived cytokinin levels have been made for wild-type *Funaria* or *Physcomitrella*, and to our knowledge, no measurements have been made to relate cytokinin levels to different stages of development. In a hybrid generated from a cross between *Funaria* and *Physcomitrium piriforme*, Beutelmann & Bauer (7) characterized the endogenous hormone and showed that its chromatographic behavior was identical to that of N^6-(Δ^2-isopentenyl)adenine (i^6Ade) and that it was found in both tissue and culture medium at $\sim$1 μM. Apart from this report, most of the information about the nature of the endogenous cytokinin responsible for bud formation has come from studies of mutants of *Physcomitrella*. Ashton et al (5) isolated and characterized a number of mutants that responded abnormally to auxin and cytokinin and have shown that a relationship exists between the presence of the hormones and bud formation. Based on their responses to added hormones, the mutants were divided into two categories, those that have altered endogenous auxin or cytokinin levels (possibly due to altered levels of synthesis, increased production of molecules that modulate hormone activity, or changes in the degradation of endogenous hormone) and those altered in their response to one or both of the hormones.

The characterization of one group of mutants that appears to have altered endogenous cytokinin levels, the bud *over*producing (*ove*) mutants, has provided evidence for the involvement of cytokinin in bud formation. 1. These mutants produce more buds than wild type on media lacking hormones, thus resembling wild-type plants treated with cytokinin (4). 2. When grown with wild-type tissue, members of one *ove* group can induce bud production in neighboring wild-type cells (4). 3. Under conditions in which the medium is continuously replaced, *ove* mutants do not produce buds; the colonies show a morphology identical to that of wild-type tissue grown under the same conditions (4). 4. Subsequent studies measured the cytokinin content of the medium from cultures of the wild type and several *ove* mutants. It was shown that the i^6Ade concentrations in the culture medium from the *ove* mutants reached a maximum of 100 nM and zeatin concentrations reached 5 nM (25, 63, 65). Cytokinin levels in the medium from the wild-type culture were approximately 1% of what was found for the *ove* mutants (65).

Very little is known about mechanisms of synthesis or catabolism of cytokinin during moss development. Evidence for cytokinin biosynthesis comes from experiments in which cultures of *ove* mutants were fed with radiolabeled adenine (62). Within hours, radiolabeled cytokinin was found in the culture

medium and in the tissue. Gerhäuser & Bopp (30) provided preliminary evidence for a cytokinin degradation pathway. They showed that *Funaria* cultures convert radiolabeled cytokinin to adenine and its derivatives.

CALCIUM AS AN INTRACELLULAR MESSENGER IN BUD ASSEMBLY

In a series of papers, Saunders & Hepler (51–53), Saunders (50), and Conrad & Hepler (15) reported experiments with *Funaria* addressing the role of calcium in bud formation. The authors concluded that calcium is involved; however, they did not determine in which process(es) it plays a role. Analysis of the data presented in these papers suggests that the studies have most often evaluated the role of calcium in the formation of the caulonema initial cell. Two observations lead to this conclusion. First, the authors often started with tissue that had not yet produced initial cells, the target cells for cytokinin-induced bud assembly. Because they added cytokinin to caulonema tissue that was not producing initial cells, this addition first induced the formation of those targets. Second, they measured the levels and distribution of intracellular calcium immediately after the addition of cytokinin, during the period of initial cell formation, before targets of bud assembly were present.

There is, however, indirect evidence suggesting that calcium in the external medium is required for bud assembly. Saunders & Hepler (52) determined the effect of artificially increasing intracellular calcium levels on initial cell formation. They found that in the absence of cytokinin, but in the presence of calcium, the calcium ionophore A23187 induced initial cell formation. When treatment with the ionophore was prolonged (under the same conditions), in some instances initial cells continued to divide and form buds with typical tetrahedral apical cells. Moreover, Markmann-Mulisch & Bopp (40) attempted to induce buds in *Funaria* in cytokinin-containing medium in which the effective concentration of calcium had been reduced using cobalt. Fewer buds formed, and those that did form were unable to undergo cell division remaining round and unicellular.

Saunders & Hepler (51) provided further evidence implicating calcium in bud assembly. They measured membrane-associated calcium at various stages of development after cytokinin addition using the fluorescent calcium-chelating probe chlorotetracycline (CTC). They found that fluorescence was four times greater in the single-celled bud than in its subtending caulonema cell, suggesting calcium levels increased during early stages of bud assembly. As bud assembly progressed, the stalk cell became highly vacuolate and less fluorescent while the dividing cells of the bud continued to display bright fluorescence at least through the formation of the tetrahedral apical cell. Using the fluorescent membrane

probe N-phenyl-1-naphthylamine (NPN) as a measure of the amount of total membrane present, some NPN fluorescence was observed in the single-celled buds, but at lower levels than observed with CTC in cells at the same stage of development. The authors concluded that the relative amount of calcium per quantity of membrane, which had increased during initial cell formation, was maintained during bud assembly. They suggested that the increases in membrane-associated calcium reflect a localized cytokinin-induced increase in intracellular free calcium.

Evidence suggesting that calcium may not be required for early events in cytokinin-induced bud assembly comes from experiments in which *Funaria* was grown in calcium-free medium (40). In this medium, initial cells swelled even in the absence of apical calcium-CTC fluorescence. The buds remained round and unicellular, suggesting that although calcium may not be required for the early events in bud assembly, it appears to be required for the subsequent cell divisions that lead to the formation of a multicellular bud.

PROSPECTS FOR THE ANALYSIS OF BUD ASSEMBLY

Early Events

While it is clear that cytokinin can stimulate the assembly of a bud from a caulonema initial cell, many questions remain about the processes involved in the cytokinin-induced signaling. For example, where is endogenous cytokinin made and where does the perception that leads to bud assembly take place? Is cytokinin made in filament cells and perceived intracellularly by the initial cell or at its plasma membrane? Do endogenous cytokinin levels change during development, or is the sensitivity of the initial cell altered in a developmentally programmed manner?

To localize endogenous cytokinin and identify sites of perception during normal development, it will be necessary to develop methods that allow detection of low levels of hormone and quantitative measurement of cytokinin in situ. In addition, information is needed about the transport of endogenous cytokinin and mechanisms of cytokinin synthesis, catabolism, or differential activation during bud assembly.

Several approaches could be used to provide information about the site of perception of exogenous cytokinin. For example, Brandes & Kende (13) monitored the distribution of radiolabeled cytokinin in *Funaria*. They treated cells with radiolabeled cytokinin and saw significant localization of radioactivity at the single-celled bud. While some radioactivity was associated with the subtending caulonema cells, little or none was localized to caulonema cells that were not or had not produced initial cells. Based on these studies, Brandes

(12) suggested that the presence of binding sites for cytokinin may be the biochemical basis for the difference between cells that form buds and those that do not. Additional experiments will be required to determine whether radiolabeled cytokinin binds to the plasma membrane or whether it is taken up into the cells. Another approach that should provide information about the site of perception of exogenous cytokinin would involve the comparison of early events in bud assembly in cells treated with impermeant cytokinin with those events in cells that have been injected with the hormone.

An initial cell that does not encounter cytokinin will form a side branch by resuming tip growth. One characteristic feature of tip growth is the presence of an oscillating gradient of calcium focused at the apex of the tip cell (43, 46). These calcium gradients may be due in part to regulated influx of calcium at the growing tip. If these gradients are disrupted (e.g. with calcium and a calcium ionophore), normal tip growth is altered (28, 39). Could a cytokinin-induced increase in calcium early in bud assembly disrupt the normal tip-focused gradient of calcium? A delocalized calcium influx might then lead to an altered distribution of organelles and vesicles as part of the change from tip growth to the pattern of expansion associated with the developing bud. An early difference between a branch or a bud then may be due to this change in calcium, and cytokinin may induce and/or maintain delocalized calcium entry.

Experiments from our laboratory have shown that a calcium transport mechanism is present on the plasma membrane in *Physcomitrella* (57–59). This transport is sensitive to 1,4-dihydropyridines (DHPs), molecules that are known to modulate calcium entry through voltage-dependent calcium channels in animal cells (14). In our studies, we have provided evidence for DHP modulation of calcium influx into moss protoplasts (57). Influx was stimulated by DHP agonists and inhibited by DHP antagonists. Calcium accumulation increased dramatically within 15 s of addition of cytokinin to protoplasts, suggesting a potential interaction of the hormone and the transporter. As has been shown for DHP-sensitive calcium transport in animal cells, this influx into moss cells was stimulated by a depolarization of the plasma membrane and was affected by numerous classes of calcium channel blockers. We have also shown that there are abundant sites for DHP binding in the *Physcomitrella* plasma membrane; DHPs bind with high affinity and specificity (58). This ligand/receptor interaction was stimulated by cytokinin at low concentrations and by heterotrimeric GTP-binding proteins (58, 59).

We are presently examining the distribution, activity, and regulation of the DHP-sensitive calcium transport activity during different stages of development. With this information, we will be able to determine whether (*a*) the transporter plays a role in initial cell formation (56), (*b*) the transporter regulates

calcium oscillations during tip growth in initial cells that have not been stimulated by cytokinin to form a bud, and (*c*) cytokinin regulation of this transporter disrupts the normal tip-focused gradient of calcium.

Determining the role of calcium in early events in cytokinin-induced bud assembly will be complicated by the apparent diversity of calcium-dependent stages during moss development. At a minimum, it will be necessary to separate the changes taking place in bud assembly immediately after cytokinin addition from processes involved in initial cell formation and from those involved in the cell divisions during later stages of bud assembly. In addition, it will be important to avoid experiments in which calcium is removed from the medium as resulting changes in bud assembly may be due to calcium's effect on other critical developmental processes. It should be possible, however, to determine whether calcium is involved in early events in cytokinin-induced bud assembly using single cell assays, which we believe represent one of the major advantages of using moss to study development. In these assays, molecules that may influence a specific process are delivered via microinjection to a particular cell at a specific stage of development. Using this approach, it will be possible to monitor cellular calcium changes immediately after the addition of cytokinin and to alter intracellular calcium levels in the initial cell in the absence of cytokinin. If calcium is involved, it will be possible to determine the timing of its involvement since bud assembly occurs progressively along cells of a single filament.

If calcium is not found to mediate these very early events in bud assembly, it will be necessary to determine how cytokinin is regulating the altered pattern of growth that takes place during the assembly of the bud. For example, what molecules are synthesized or activated in response to cytokinin? How do they lead to the changes in cell expansion and elongation seen early in bud assembly or to the cell divisions and subsequent morphological changes that result in formation of the multicellular bud? Is cytokinin causing structural changes by regulating the rate of wall synthesis and degree of wall extensibility as the initial cell swells? Does it alter the stability or biochemical composition of the cytoskeleton to allow for changes in deposition of wall and membrane material during initial cell swelling or the orientation of the mitotic apparatus during subsequent cell divisions?

Later Events

The processes that result in daughter cells with different fates are fundamental to the generation of cell diversity during development (37). During cytokinin-induced bud assembly, the initial cell reaches a specific size and then undergoes an asymmetric division to produce two cells of different sizes with very different fates. This asymmetric division must require the coordination of two events: establishment of cytoplasmic polarity and orientation of the mitotic apparatus along the axis of polarity. A number of questions need to be answered if we

are to understand how these processes take place. For example, what cell fate determinants are distributed differentially to the daughter cells? Do polarized components of the cytoskeleton provide a structural basis for localizing these determinants? Which genes play a role in the regulation of the asymmetric division? How are these genes regulated and how do the resulting gene products act?

Asymmetric divisions are common in many organisms, and genes that are responsible for these divisions, and the subsequent cell fate, have been identified in plants, nematodes, insects, yeast, and bacteria (1, 20–22, 31, 34, 49). Recognizing that multiple mechanisms may be responsible for the generation of asymmetry, careful comparative studies of asymmetric division in numerous organisms may suggest candidate molecules that may underlie this process in moss. Once these molecules are identified, it should be possible to determine whether homologs exist in moss. For example, conserved sequences found in genes in these other organisms could be used as hybridization probes or primers for polymerase chain reaction. Methods that enable isolation of cDNAs from moss that functionally complement asymmetry defects in other organisms also offer a potentially powerful route for identifying homologs of molecules important in this process. In vivo assays altering the levels and distribution of putative regulatory molecules before, during, and after asymmetric division of the initial cell should provide important insights into the role and regulation of these molecules during bud assembly.

Studies have shown that cytokinin is not just a trigger for bud assembly; its presence is required for several hours to prevent reversion to normal filament growth (13). In experiments using a photolabile cytokinin, Sussman & Kende (61) showed that reversion can be induced by exposing the tissue to UV light to destroy the cytokinin, as well as by washing the tissue (13). Since it is not clear if the caulonema filaments used as starting tissue for these studies were producing initial cells, it is not possible to determine the duration of cytokinin exposure required for bud assembly alone. However, as was shown by the formation of a filament from a multicellular bud after cytokinin removal, clearly some prolonged exposure to cytokinin is required to commit growth to bud assembly. A number of questions arise concerning this prolonged requirement for cytokinin. Is continued exposure to cytokinin required to ensure production of sufficient levels of a product that is required for commitment to bud assembly? Is cytokinin required at multiple steps in the pathway of a cytokinin-induced cascade?

GENETIC ANALYSES OF BUD ASSEMBLY

The studies outlined above have begun to provide insight into the events that take place during cytokinin-induced bud assembly and some of the mechanisms involved. Continued progress toward understanding the underlying biochemical and molecular mechanisms will be facilitated by thorough characterization

of previously identified developmental mutants. Progress will also be aided by the identification of additional relevant mutations, such as those leading to altered perception and transduction of the cytokinin signal or altered levels of downstream interacting components. Potentially, mutants will allow the identification of genes important in these processes. In addition, simple mutations can be used in conjunction with the cellular and molecular tools currently available to characterize the underlying defects. In the following section, we describe the current status of genetic analyses in moss and identify technological advances that will be required for genetics to further contribute to our understanding of mechanisms underlying development in this organism.

Most genetic studies have been performed with *Physcomitrella*. Recent estimates suggest that in the wild type $n = 27$, and the DNA content is 0.6 pg per haploid genome corresponding to 600 megabase pairs (48). Sporophytes can arise as a result of either self- or cross-fertilization via union of gametes from gametophytes produced from spores of the same or different sporophytes. Wild-type strains are normally self-fertile. In culture, *Physcomitrella* has a short generation time of approximately 12 weeks.

Conditions have been described for the isolation and characterization of morphological and amino acid and purine analog-resistant mutants (2, 4, 5). Mutations have been induced in haploid spores or filament cells with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG), ethylmethane sulphonate (EMS), or ultraviolet light. During somatic mutagenesis, filament tissue is treated with a mutagen, and protoplasts are isolated (64). Regeneration of protoplasts leads to the formation of tissue that can be screened for mutant phenotypes. Following mutagenesis of either spores or filament cells, mutant strains have been isolated most often using nonselective isolation (47). With this approach, the mutagenized protoplasts or spores are cultured initially on medium that will promote filamentous growth. After 1–2 weeks of growth, the tissue is transferred to conditions that select for the desired mutant phenotype; putative mutants can usually be identified within 2–3 weeks. Male and female gametes borne on the same individual gametophyte are genetically identical. Thus, sporophytes arising after fusion of these gametes are homozygous for the induced mutation, and all resulting spores contain the mutation. If strains are fertile, classical techniques can be used for complementation analysis, dominance testing in nonhaploid tissue, and linkage studies.

It is easy to identify characteristics of moss that make it well suited for genetic analyses. The production of single-celled spores and subsequent development of the prolonged gametophytic stage allow genetic studies at the haploid level. As described above, it is possible to mutagenize spores and identify gametophytic mutations. In addition, self-fertilization of gametophytes results in completely homozygous sporophytes. Because moss can be propagated

vegetatively, mutants whose terminal phenotypes are expressed at a later stage in development can often be maintained at an earlier stage (23).

Some of the same characteristics that make moss well suited for genetic analyses also present limitations. The prolonged and complex, multicellular haploid stage means that for a greater portion of the moss life cycle, lethal mutations cannot be masked by a wild-type allele, as would be possible in a diploid. In addition, genetic analyses have been hampered by the discovery that certain mutant strains possess alleles that are dominant or incompletely dominant to their respective wild-type alleles (5).

One of the greatest limitations to genetic studies in moss has been the fact that many of the developmentally abnormal mutants are sterile (32). Somatic hybridization following protoplast fusion has been used to circumvent the sterility that is a consequence of mutants whose development is blocked prior to gamete production (5, 26, 32, 33). In this procedure, protoplasts made from filamentous tissue of one (mutant) strain are mixed with protoplasts prepared from another strain. Cellular and nuclear fusion are induced using chemical (5, 26, 32, 33) or electrical (66) methods. The protoplasts then regenerate into filamentous gametophytes under conditions that select for hybrids. While karyotypic analyses have not been reported, segregation ratios of progeny resulting from self-fertilization of such hybrids suggest that most of the hybrids are diploid (17, 32). Possibly due to the change in gene dosage, the morphologies of the somatic hybrids are variable and are unlike the morphologies of the parental haploid strains.

Somatic hybrids have generally been found to have low rates of reversion to the wild-type phenotype, and the hybrid phenotypes are stable after numerous subcultures. These hybrids can be used for more detailed genetic analysis if sporophyte production occurs. Since many of the hybrids have been found to produce fewer sporophytes than wild type or to be sterile, they have been used most often in complementation analyses (26, 33). For example, using somatic hybridization, it has been shown that one group of *ove* mutants occurs relatively frequently, is recessive to wild type, and is associated with at least three complementation groups (26).

IMMEDIATE GOALS FOR MOSS RESEARCH

Hormone-induced bud assembly in moss provides a unique opportunity to study differentiation, morphogenesis, and organogenesis in vivo in a relatively simple organism. The advantages of using moss for these studies include the following: 1. The process of bud assembly is well defined and normal development can be manipulated experimentally (13). 2. The moss has only a few cell types, so it is possible to isolate the events in bud assembly from other developmental events. 3. Because bud assembly occurs progressively along

a filament, it should be possible to determine when and where molecules involved in this process are important. 4. The multicellular gametophyte enables the study of more complex development than is possible with most other haploid organisms. 5. The cells involved are large and accessible and, as a result, are amenable to testing the in vivo function of molecules using microinjection technology. 6. Using liquid cultures of *Funaria* that are enriched for specific stages of development (38), it is possible to produce stage-specific tissue for biochemical, cellular, and molecular biological analyses. 7. Moss cells have been successfully transformed (55; K Schumaker, unpublished results); this should provide an additional approach with which to study gene function during development. For example, this can be done in transformation experiments in which the levels of a gene product are altered in the wild type. Alternatively, a mutant phenotype may suggest candidate molecules that can rescue the mutant; transformation will provide a functional assay for such molecules. Successful transformations have used polyethylene glycol-mediated uptake of DNA into protoplasts. Protoplast regeneration takes approximately one week and always leads to the production of chloronema filaments first, indicating that the developmental program appears to reset at this stage. As a result, it will be necessary to isolate stage-specific promoters in order to study expression and function of cloned genes at particular stages during development. Preliminary experiments examining the differential patterns of mRNA expression during development suggest that isolation of stage-specific promoters will be feasible (K Schumaker & M Dietrich, unpublished data). 8. A recent report describes homologous recombination in *Physcomitrella* (54). The authors have provided evidence for tandem insertions at several independent, targeted sites; this will be extremely useful for producing null mutations. Further refinements of this technique, resulting in gene replacement, will make this an invaluable tool for moss research.

From whole plant, cellular, and subcellular studies over the past 20 years, we know that bud formation in moss involves in sequence: differentiation of a chloronema tip cell into a caulonema tip cell, production of caulonema filament cells, caulonema initial cell formation, and cytokinin-induced assembly of the bud from the caulonema initial cell. In order to understand the mechanisms underlying these processes, two overriding goals will need to be met. It is critical to experimentally isolate the specific process under study. Studies of bud formation that do not distinguish initial cell formation and bud assembly will not permit an understanding of the underlying mechanisms of either process and will produce results that are difficult to interpret. Of equal importance is the need to standardize the protocols used for culturing moss tissue. This will allow research using the most appropriate tissue for the specific developmental event and permit meaningful evaluation of the data among laboratories.

Once these major goals have been met, where should the focus of research with moss be directed? While we anticipate that significant progress will be made in answering many of the questions outlined above, we believe that the identification and characterization of additional developmental mutants should be an immediate focus of research. Generation of these mutants will ultimately allow identification of novel molecules critical for the processes underlying bud assembly.

Generation of Additional Developmental Mutants

CONDITIONAL MUTANTS It is critical that a strategy be developed to identify mutants in which expression of the mutation can be controlled, for example, by isolation of developmentally abnormal mutants that are temperature-, pH-, or calcium-sensitive (45, 47). Conditional mutants would be an extremely powerful tool for the study of protein function in vivo, as they provide a reversible mechanism with which to lower the level of a specific gene product at any stage during growth simply by changing the growth conditions. Isolation of conditional mutants will be beneficial because many of the moss developmental mutants are sterile. In addition, such an approach might enable the identification and characterization of mutations that would otherwise be lethal. If the mutations are extreme and if moss genomes do not include redundant or alternate gene products to assume the function of the altered gene, nonconditional mutations in genes essential for development might otherwise never be identified.

Since there is currently no general method to predict which mutations in a protein will give rise to a conditional phenotype, mutants must be generated by random mutagenesis followed by screening for conditional phenotypes. This approach should work well with moss due to its relatively simple morphology, the ease of screening tissue derived from a large number of spores or protoplasts, and its short generation time in culture. Isolation of temperature-sensitive mutations should be especially feasible in *Funaria* based on the relatively wide range of temperatures in which normal wild-type growth occurs (10°C to greater than 25°C). There is one report in the literature describing the isolation of a temperature-sensitive developmental mutant in *Physcomitrella* (29). This mutant, *ove* 409, produces leafy gametophytes with wild-type morphology when maintained at low temperature. As the temperature is increased, the phenotype changes to that of a bud-overproducing mutant, suggesting that the mutant may be temperature-sensitive for cytokinin production. However, further analysis of media from wild type, a nontemperature-sensitive *ove* mutant, and *ove* 409 showed that all contain more cytokinin at elevated temperatures. The authors suggested that although the mutant is temperature-sensitive for bud production, the allele might not encode a temperature-sensitive gene product. Rather, the *ove* phenotype at higher temperatures might be due to the production of

cytokinin at levels high enough to increase bud formation. Effort in isolating conditional mutants will be fundamental to the identification of critical defects in essential functions that, at the same time, allow maintenance of the mutant strain.

INSERTIONAL MUTANTS Insertional mutagenesis has become an important approach for the identification of developmental mutants in plants (6, 27). This general strategy allows insertion of known sequences at random sites in the genome to disrupt the function of unknown genes and create a molecular tag for subsequent gene isolation. Isolation, cloning, sequencing, and further analysis of the developmental gene affected are then feasible. Success with this approach in moss will require the identification of endogenous transposable elements or introduction of foreign elements through transformation.

CONCLUDING REMARKS

The goals of this review were to describe what is known about early events in bud assembly and to evaluate the advantages and limitations of studying moss to understand hormone-induced changes that take place during development. It is clear that moss has great potential as an experimental organism for understanding the underlying physiological processes using biochemical, cellular, and molecular biological approaches. We have identified several objectives that, once achieved, will enable moss to reach its full potential as a model for developmental studies. Some of these objectives, such as defining the stages of development under study and standardizing the methods of tissue growth, should be relatively easy to meet. Others, such as developing the tools required for routine genetic analyses, will require a more significant commitment of time and resources. The effort will be worthwhile, since many of the developmental changes taking place during cytokinin-induced bud assembly in moss appear to be common to other more complex organisms. Therefore, results from studies with moss should provide important information about mechanisms underlying hormone-induced development in general.

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Volume 49, 1998

CONTENTS

THEMES IN PLANT DEVELOPMENT, *Ian Sussex* 0
GENETIC ANALYSIS OF OVULE DEVELOPMENT, *C. S. Gasser, J. Broadhvest, B. A. Hauser* 1
POSTTRANSLATIONAL ASSEMBLY OF PHOTOSYNTHETIC METALLOPROTEINS, *Sabeeha Merchant, Beth Welty Dreyfuss* 25
BIOSYNTHESIS AND FUNCTION OF THE SULFOLIPID SULFOQUINOVOSYL DIACYLGLYCEROL, *Christoph Benning* 53
SPLICE SITE SELECTION IN PLANT PRE-mRNA SPLICING, *J. W. S. Brown, C. G. Simpson* 77
PROTEIN TARGETING TO THE THYLAKOID MEMBRANE, *Danny J. Schnell* 97
PLANT TRANSCRIPTION FACTOR STUDIES, *C. Schwechheimer, M. Zourelidou, M. W. Bevan* 127
LESSONS FROM SEQUENCING OF THE GENOME OF A UNICELLULAR CYANOBACTERIUM, *SYNECHOCYSTIS* SP. PCC6803, *H. Kotani, S. Tabata* 151
ELABORATION OF BODY PLAN AND PHASE CHANGE DURING DEVELOPMENT OF *ACETABULARIA* : How Is the Complex Architecture of a Giant Unicell Built, *Dina F. Mandoli* 173
ABSCISIC ACID SIGNAL TRANSDUCTION, *Jeffrey Leung, Jérôme Giraudat* 199
DNA METHYLATION IN PLANTS, *E. J. Finnegan, R. K. Genger, W. J. Peacock, E. S. Dennis* 223
ASCORBATE AND GLUTATHIONE: Keeping Active Oxygen Under Control , *Graham Noctor, Christine H. Foyer* 249
PLANT CELL WALL PROTEINS, *Gladys I. Cassab* 281
MOLECULAR-GENETIC ANALYSIS OF PLANT CYTOCHROME P450-DEPENDENT MONOOXYGENASES , *Clint Chapple* 311
GENETIC CONTROL OF FLOWERING TIME IN ARABIDOPSIS, *Maarten Koornneef, Carlos Alonso-Blanco, Anton J. M. Peeters, Wim Soppe* 345
MEIOTIC CHROMOSOME ORGANIZATION AND SEGREGATION IN PLANTS, *R. Kelly Dawe* 371
PHOTOSYNTHETIC CYTOCHROMES *c* IN CYANOBACTERIA, ALGAE, AND PLANTS, *Cheryl A. Kerfeld, David W. Krogmann* 397
BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development, *Steven D. Clouse, Jenneth M. Sasse* 427
NUCLEAR CONTROL OF PLASTID AND MITOCHONDRIAL DEVELOPMENT IN HIGHER PLANTS, *P. Leon, A. Arroyo, S. Mackenzie* 453
BORON IN PLANT STRUCTURE AND FUNCTION, *Dale G. Blevins, Krystyna M. Lukaszewski* 481
HORMONE-INDUCED SIGNALING DURING MOSS DEVELOPMENT, *Karen S. Schumaker, Margaret A. Dietrich* 501
EVOLUTION OF LIGHT-REGULATED PLANT PROMOTERS, *Gerardo Argüello-Astorga, Luis Herrera-Estrella* 525
GENES AND ENZYMES OF CAROTENOID BIOSYNTHESIS IN PLANTS, *F. X. Cunningham Jr., E. Gantt* 557

RECENT ADVANCES IN UNDERSTANDING LIGNIN BIOSYNTHESIS, *Ross W. Whetten, John J. MacKay, Ronald R. Sederoff* 585
DESATURATION AND RELATED MODIFICATIONS OF FATTY ACIDS, *John Shanklin, Edgar B. Cahoon* 611
PHYTOREMEDIATION, *D. E. Salt, R. D. Smith, I. Raskin* 643
MOLECULAR BIOLOGY OF CATION TRANSPORT IN PLANTS, *Tama Christine Fox, Mary Lou Guerinot* 669
CALMODULIN AND CALMODULIN-BINDING PROTEINS IN PLANTS, *Raymond E. Zielinski* 697
FROM VACUOLAR GS-X PUMPS TO MULTISPECIFIC ABC TRANSPORTERS , *Philip A. Rea, Ze-Sheng Li, Yu-Ping Lu, Yolanda M. Drozdowicz, Enrico Martinoia* 727