CHATTARAJ, PARNA. Gravitropism in *Physcomitrella patens* : A Microtubule Dependent Process (Under the direction of Dr. Nina Strömgren Allen)

The plant cytoskeleton plays an important role in the early stages of gravisignaling (Kiss, 2000). Although in vascular plants, actin filaments are used predominantly to sense changes in the gravity vector, microtubules have been shown to play an important role in moss gravitropism (Schwuchow et al., 1990). The moss *Physcomitrella patens* is a model organism and was used here to investigate the role of microtubules with respect to the gravitropic response. Dark grown caulonemal filaments of *P. patens* are negatively gravitropic and the readily imaged tip growing apical cell is a "single-cell system" which both senses and responds to changes in the gravity vector. MTs were imaged before and after gravistimulation with and without MT depolymerizing agents. Six-day-old filaments were embedded in low melting agarose under dim green light, allowed to recover overnight in darkness and gravistimulated for 15, 30, 60 and 120 min. Using indirect immunofluorecence and high resolution imaging, MTs were seen to accumulate in the lower flank of the gravistimulated tip cell starting 30 min post turning and peaking 60 min after gravistimulation of the cells. The microtubule depolymerizing drug, oryzalin (0.1 µM for 5 min), caused MTs to disintegrate and delayed MT redistribution by 3hrs 30min. Growth of the oryzalin treated filaments was analyzed and a delay in growth was observed for both gravi and non-gravistimulated filaments. Tip cells bulged and sometimes branched after 75 min. This study demonstrates that microtubules are important for growth in *P. patens* and MT depolymerization leads to a delayed growth and graviresponse.

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GRAVITROPISM IN *PHYSCOMITRELLA PATENS* : A MICROTUBULE DEPENDENT PROCESS

by

PARNA CHATTARAJ

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APPROVED BY:

Margaret E Daub
Committee member

Wendy F Boss
Committee member

Nina Strömgren Allen
Chair of Advisory Committee

DEDICATION

This thesis is dedicated to my husband and my parents who have always been with me through these years and whose support and well wishes inspired me to become a researcher.

PERSONAL BIOGRAPHY

I was born in a small town of India and went to St Agnes Elementary and Middle School. From my childhood I liked nature but my interest in biology was generated by my schoolteacher Ms Basantika during 6th grade. She was a great teacher, who taught biology with an interesting touch. I graduated from my high school, Hijli High in Kharagpur, India and moved to the metropolitan city of Calcutta where I attended the University of Calcutta, India and received B.Sc. and M.Sc degrees in Botany. My M.Sc included a comprehensive literature review on Bacterial Canker of Citrus. I married and moved to the USA in 1999. After arriving here I became a lab technician in the RTP division of the biotech company Novartis (Syngenta) for 6 months. This job gave me the important experience of working in the middle of a research community. In order to enhance this experience I joined North Carolina State University where I have been working in Dr. Nina Allen's laboratory these past years. Working in this laboratory has been a great experience for me especially in the field of imaging and I hope to make use of this invaluable knowledge in my future.

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LIST OF ABREVIATIONS

Abbreviation	
ABP	Actin Binding Protein
DIC	Differential Interference Contrast
ER	Endoplasmic reticulum
F-actin	Filamentous actin
G-actin	Globular actin
HGMF	High Gradient Magnetic Field
IAA	Indole-3-acetic acid
I_L	Intensity of lower flank
I_U	Intensity of upper flank
IP_3	Inositol 1,4,5-triphosphate
IR	Infra Red
kDa	Kilodalton
MAP	Microtubule Associated Protein
MT	Microtubule
min	Minute
mM	Millimolar
N.A.	Numerical aperture
nm	Nanometer
PBS	Phosphate Buffered Saline
pH	Negative log of the hydrogen ion concentration
S	Second
UV	Ultraviolet
µm	Micrometer

1 INTRODUCTION

1.1 Gravitropism in Plants

All organisms must in order to survive perceive and respond to many different environmental stimuli. Plants, which are stationary, are no exception and they must be able to sense and respond to these cues in a great variety of ways. Gravity is one of the most important stimuli affecting plant growth and development. The growth of plants and plant organs in response to the direction of gravity is known as gravitropism (Kiss, 2000). In higher plants, roots grow towards gravity (i.e. they are positively gravitropic) while shoots grow away from gravity (i.e. they are negatively gravitropic). Gravitropism plays an important role in single cell systems and has also evolved to the systems level in higher plants. Two examples of cells which respond to a gravitropic stimulus are: *Chara* (algae) rhizoids (Sievers and Volkmann, 1979) and the apical protonemal cell of the mosses *Ceratodon*, *Physcomitrella* and *Funaria* (Hartmann, 1984; Jenkins et al., 1986; Schwuchow et al., 1995). These systems are multicellular and filamentous but the gravi-sensing and response occur only in the tip-growing apical cell (Young and Sack, 1992). Different aspects of gravitropism have been reviewed by: Björkman, (1988), Sack and Kiss (1989), Sack (1991), Masson (1995), Masson et al., (2002), Sack (1997), Chen et al., (1999), Hasenstein et al., (1999) and Ranjeva et al., (1999). Gravitropism can be divided into three steps: sensing, transduction and response (Sack, 1991). Sensing is the detection of the gravity signal. This is followed by signal transduction during which the detected signal is communicated to a targeted organ or cell and the response is the observed alteration in growth of the targeted organ. Fig 1.1 illustrates the 3 stages of gravitropism.

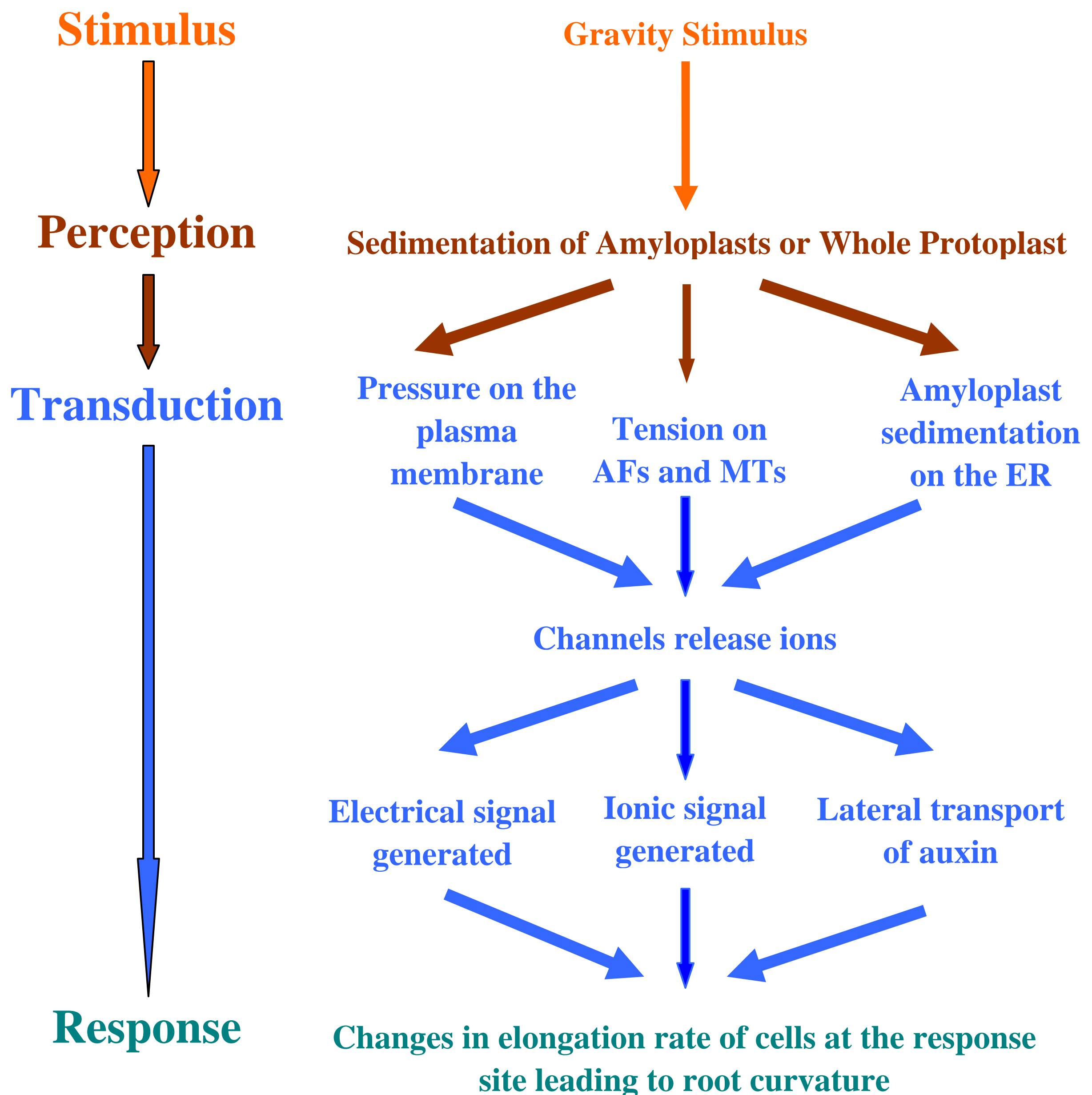

Fig 1.1 Different stages of gravitropism.

Diagrammatic representation of what is known about different stages of gravitropism and the involvement of various cellular components like the cytoskeleton, the plasma membrane, the ER, ions, hormones etc. in the gravitropic pathway.

1.1.1 Perception of gravity by plants

In general, gravity is physically sensed by a cellular component, called the susceptor (Björkman, 1988). The mass of the susceptor falls on the receptor, which turns on the signal transduction phase. The susceptor and receptor are located in the same cell in plants. The cells that harbor the susceptor and receptor are known as statocytes (Björkman, 1988).

1.1.1.1 Location of statocyte

Gravity is sensed in different tissues of roots and shoots in higher plants. Darwin (1881) recognized that when root caps were removed, roots failed to respond to gravity and he concluded that the root cap was the probable sensory site of for a gravistimulus in roots. Other tissues where gravisensing occurs include stems, primary and lateral roots, leaf and stem pulvini, hypocotyls and inflorescence stems. In most cases the perceiving cells in that organ contains amyloplasts.

In algae and mosses gravity is sensed by one specialized cell. Rhizoids of the green alga *Chara* are positively gravitropic, whereas *Chara* protonema are negatively gravitropic (Braun, 1997). The moss protonemata is a filamentous structure that germinates from a haploid spore (Cove et al., 1997). The life cycle of the moss *Physcomitrella patens* includes a free living haploid gametophyte and a diploid sporophyte (Fig. 1.2). This system is advantageous in that the, responses of both multicellular gametophores and unicellular protonemata can be observed in the same system (Jenkins et al., 1986). In darkness, both the protonemata (Fig 1.3) and the gametophores are negatively gravitropic and only the tip growing apical cell {Fig 1.4

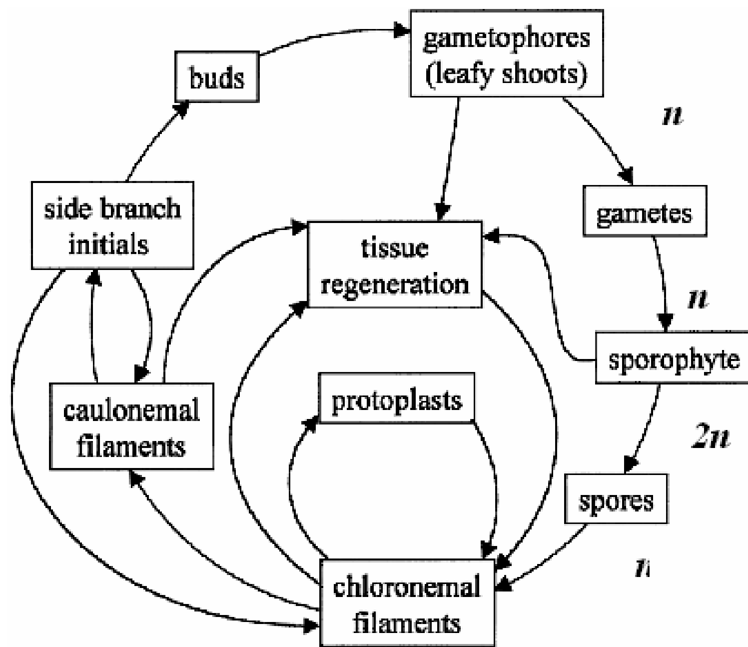

Fig 1.2 Life cycle of *P. patens*

Life cycle of *P. patens* alternates between two major phases: a free living haploid gametophyte and a diploid sporophyte. This study used caulonemal filaments. (Modified from Cove, J Plant Growth Regul, 2000).

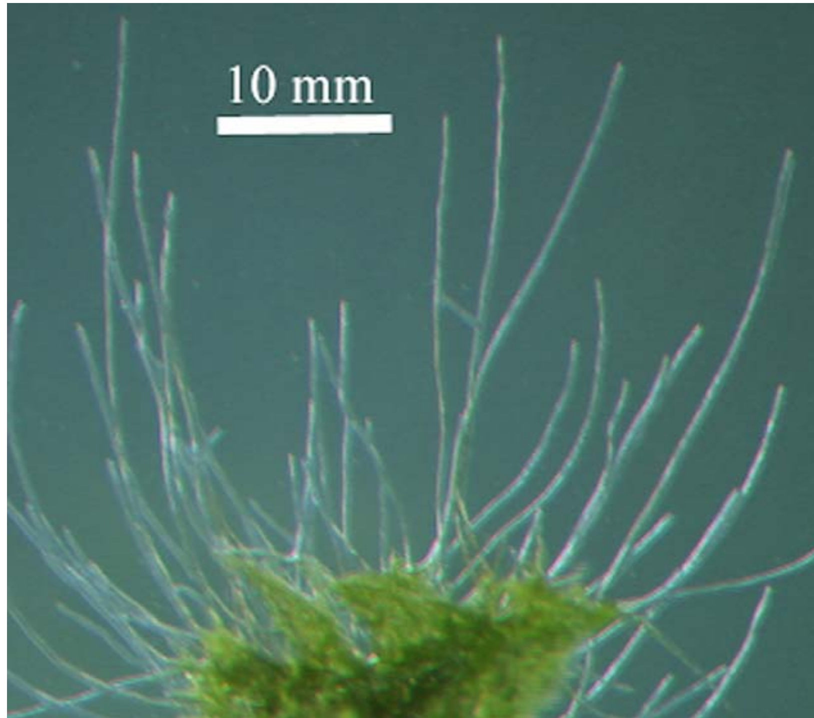

Fig 1.3 Caulonemal filaments grown in darkness.

Vertically growing, negatively gravitropic caulonemal filaments arising from protonemal fragments in darkness (Courtesy Eva Johannes). Bar = 10 mm.

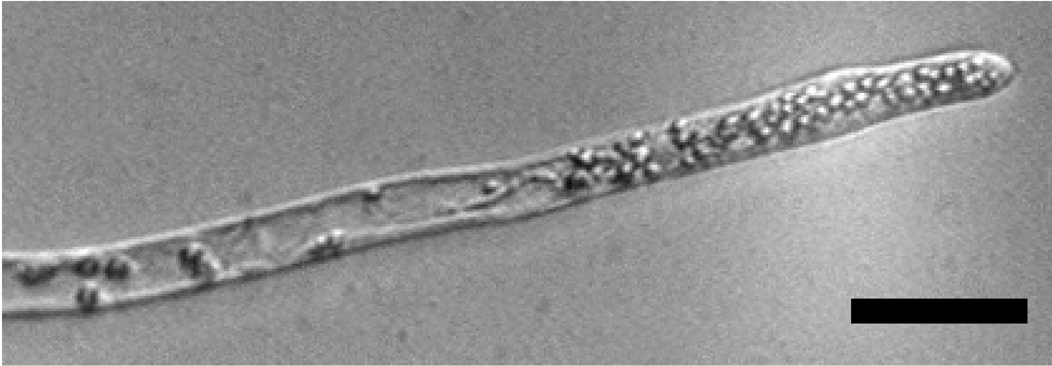

Fig 1.4 Gravitropic response of an apical cell of a caulonemal filament.

An apical cell of a caulonemal filament responding to gravity. The moss was rotated 90 degrees and kept at the same angle for approximately 10 hours in the dark. It grew slowly upwards. Amyloplasts are seen in an area below the tip. A large vacuole is seen in the left area of the cell image. Bar = 50 μm.

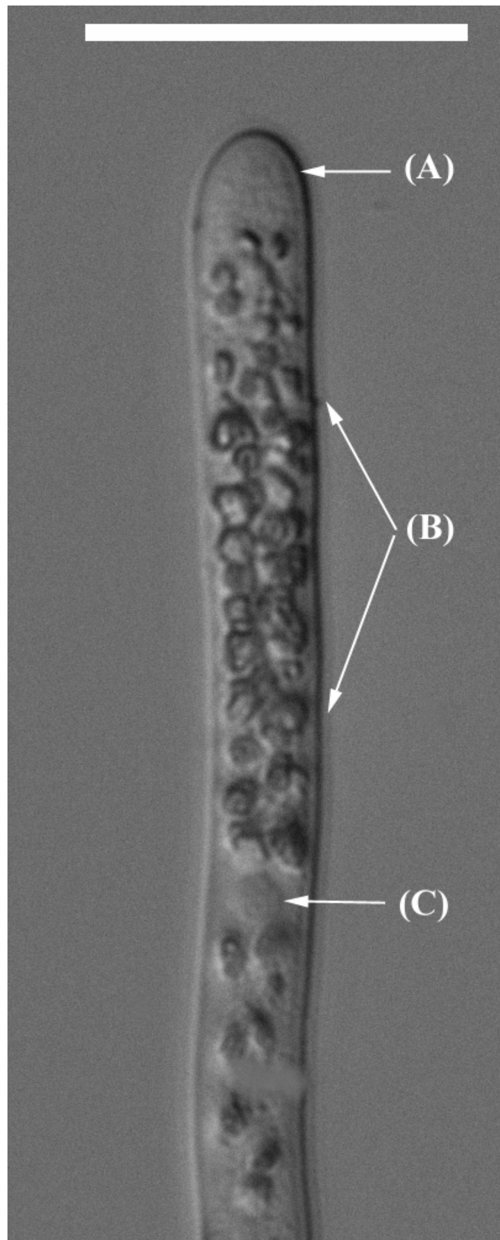

Fig 1.5 Caulonemal tip cell displaying prominent amyloplasts.

A vertically grown apical caulonemal cell displaying an amyloplast free zone at the tip (**A**) followed by a dense amyloplast zone (**B**) and the nucleus (**C**). Bar = 50 µm.

(gravistimulating filament), Fig. 1.5 (vertically growing filament)} of the protonemata senses changes in the gravity vector.

1.1.1.2 The Susceptor

The susceptor senses gravity at the cellular level. Sack, (1991) proposed 5 different types of susceptors:

1. Statoliths, which are dense, sedimenting e.g. amyloplasts in root columella cells.
2. The entire protoplast e.g. in *Chara* internodal cells,
3. High density cellular components that stretch and/or compress without sedimentation e.g. cytoskeletal elements
4. Intracellular components that rise and
5. Extracellular components located at or outside the plasmalemma.

According to Björkman (1988) a gravity sensor may be present inside or outside the cell allowing the cell to sense its motion, displacement or position relative to the gravity vector. An effective sensor should be relatively insensitive to random thermal energy but very sensitive to changes in the direction of gravity (Björkman, 1988). The sensor needs to overcome the thermal noise and induce a force that will stimulate a gravi-response.

The release of this force/energy must occur within a time frame so that the signal is sent prior to the presentation time. The time to make a commitment for a minimal growth response after gravistimulation is called the presentation time and appears to be a measure of gravitropic sensitivity (Björkman, 1988; Kiss, 2000)). A great deal of information exists about the presentation time of gravistimulated *Arabidopsis thaliana.*

The presentation time of a normal wild type *Arabidopsis* root is 0.4 min (Kiss, 2000) and it can be very short (about 7s) at 1g in *Lepidium* roots (Larsen, 1979). Presentation time in the moss *Ceratodon* is between 12- 17 min (Walker and Sack, 1990).

There are two major models for gravity perception in plants (Sack, 1997): The starch-statolith hypothesis and the gravitational pressure hypothesis. Both have merit and I will describe each in turn.

1.1.1.2.1 The starch-statolith hypothesis

This classical theory of gravity perception was proposed by Haberlandt and Nemec (1900) and still is one of the most accepted hypothesis for how higher plants as well as mosses sense gravity. According to this theory, specialized dense starch filled organelles called amyloplasts function as statoliths (Sack, 1991). As the amyloplasts sediment, they somehow trigger the transduction of the stimulus. The sedimentation occurs in special cells, at specific areas and in a distinct developmental phase (Sack, 1991). In order to act as statoliths, the amyloplasts must overcome the background thermal noise. According to Björkman (1988), the approximate potential energy produced from a single root cap amyloplast displaced by gravity is about 250 times the thermal noise and about 15 times the activation energy predicted for a gravi-perception.

In roots, amyloplast sedimentation occurs in the central columella cells of the root cap (Sack and Kiss, 1989; Sievers and Braun, 1996) while in shoots they sediment in the endodermis (Sack, 1987) and in pulvini of maize and other grasses the sedimentation occurs in bundle-sheath cells (Johannes et al., 2001). In mosses, the amyloplasts are very prominent in the caulonemal tip cells and the sedimentation occurs in their sub apical zone (Sack, 1997). *P. patens* also has an amyloplast region in the sub apical zone. Fig 1.4

illustrates a gravistimulated filament and Fig. 1.5 illustrates the distribution of amyloplasts, vacuoles and cytoplasm in part of a vertically grown *P. patens* tip filament. In *Ceratodon*, unlike columella cells, only a subset of amyloplasts undergoes sedimentation (Sack et al., 2001). Amyloplast sedimentation starts before the upward curvature occurs in *Ceratodon* supporting the hypothesis that sedimentation triggers the response (Walker and Sack, 1990). Walker and Sack (1991) showed that if amyloplasts in *Ceratodon* are removed from the tip of the apical cell by basipetal centrifugation, the horizontal protonemata do not gravistimulate until the amyloplasts return to their normal position where they then can sediment. Sedimentation of amyloplasts is a common feature among different species of mosses but the degree of sedimentation varies. In *Ceratodon* the sedimentation is very obvious (Sack et al., 2001) and starts within 15 min of gravistimulation (Walker and Sack, 1990) while in *Physcomitrella* it is more subtle (Schwuchow et al., 1995). Recently, Schwuchow et al., (2002) have shown that the lateral sedimentation zone is conserved in about nine gravitropic mosses, implying that the sedimentation has a specialized and conserved function in gravitropism (Schwuchow et al., 2002). In *Ceratodon*, the protonemata show negative gravitropism even when they are immersed in a media denser than the apical cell (Schwuchow et al., 2002). This means that the gravi-sensing is achieved by using an intracellular component in the protonemata (Schwuchow et al., 2002). In *Ceratodon*, sedimentation of amyloplasts by a high gradient magnetic field (HGMF) induced curvature towards the field (Kuznetsov et al., 1999). HGMF mimicks gravitropism (i.e with sufficient intensity and magnetic gradient it can displace amyloplasts), indicating that amyloplast displacement is important for gravity sensing (Sack et al., 2001). All these observations strongly support

a statolith based sensing system in mosses.

In *Chara* rhizoids, statoliths play an important role in gravitropism. Tips of *Chara* rhizoids contain about 50 small, highly refractive vesicles containing $BaSO_4$ (Schroter et al., 1975; Sievers et al., 1996). During horizontal stimulation, complete sedimentation of statoliths to the lower flank occurs within 15 min (Sievers and Schröter, 1971) Reduction in numbers of these vesicles by immersing rhizoids in artificial media reduced gravitropism drastically (Kiss, 1994). Displacement of the $BaSO_4$ particles following manipulation with optical tweezers caused a tip reorientation – corresponding to positive gravitropism (Leitz et al., 1995; Braun, 2002).

1.1.1.2.1.1 Reduced sensitivity of starchless and starch-deficient mutants

The starch-statolith hypothesis is receiving support from experiments in which starch is removed by chemical treatments or by producing different types of starchless/ starch-deficient mutants. Destarching tissues by chemical methods may harm them to such an extent that they become gravi-insensitive though the response is not related to starch removal (Sack, 1991). There are starchless mutants that are less sensitive to gravitropism. *Arabidopsis* mutants TC7 and ACG21 are defective in plastidic phosphoglucomutase (*pgm*) and have significantly smaller gravitropic responses than the wild type (Casper and Pickard 1989, Kiss et al., 1996). Three other mutants (NS 458 of *Nicotiana sylvestris* is almost starchless, ACG 27 of *Arabidopsis* has 60% of wild type starch while ACG 20 has about 51% of wild-type starch) exhibit various degree of reduced gravitropism (Sack, 1997). According to Caspar, (1994), the reductions of gravi-sensitivity in the mutants are probably not related to alterations in carbohydrate metabolism, since although the gravitropism is reduced, growth rates are normal. Hence

the reduced gravitropism is probably due to a reduction in sensing of the gravity change (Sack, 1997). Clearly, the amyloplast settling does play a role in sensing gravity and is necessary to get the full gravitropic response even though gravitropic sensing can occur without starch and is not absolutely required for sensing (Sack, 1997) except in *Arabidopsis* floral stalks. Maybe there are other sensors of gravity in a cell and amyloplasts are but one of these systems. When one sensor does not work, another could take over the sensing mechanism and help respond to the stimulus. This could possibly contribute to the reduced gravitropism in stachless and starch deficient mutants.

The best support for the starch-statolith theory comes from shoot mutants. Shoot gravitropic mutants sgr1 and sgr7 (Fukaki et al., 1998) are insensitive to gravity and lack sedimentable plastids in the shoots. Their growth rates however are normal and their roots are gravitropic with sedimenting amyloplasts.

1.1.1.2.2 Gravitational Pressure Model

This theory was proposed earlier by Pfeffer in 1881 (referenced in Wayne et al., 1990) and more recently has been based on the gravity-induced polarity of cytoplasmic streaming in the internodal cells of *Chara* (Wayne et al., 1990). Internodal cells of Characean algae are large and exhibit a highly organized and rapid rotational cytoplasmic streaming (Corti, 1774). Vertical internodal cells possess a polarity in cytoplasmic streaming where the motion of the downward stream is about 10% faster than the upward stream and the polar ratio {polar ratio is the ratio of velocity of downward cytoplasmic streaming to upward cytoplasmic streaming in a vertical internodal cell (Wayne et al., 1990)} of the cell is ~ 1.10 (Staves et al., 1992, 1995). In *Chara*, the polarity of cytoplasmic streaming disappears when the polar ratio = 1, while the polarity of

streaming reverses when the polar ratio < 1 (Staves et al., 1997a). Calculations show that the energy produced by the settling protoplast in *Chara* is about 10^6 times larger than the background noise and is enough to open Ca^{+2} channels (Wayne et al., 1990).

According to this hypothesis, the entire protoplast senses gravity, and the ability of a cell to sense gravity depends on its static buoyancy (Staves, 1997). So, in a less dense medium a cell's protoplast will settle on the cell wall or on the extracellular matrix, "producing a differential tension and compression between the plasma membrane and the extracellular matrix at the top and bottom of the cell, respectively" (Staves, 1997). Gravireceptors residing at the lower and upper part of the cell may get activated by these differential pressures (Wayne et al., 1990). According to Staves (1997), when the density of external medium is the same as that of the protoplast, the protoplast will attain neutral buoyancy and will not settle and no gravi-response will occur (polar ratio =1). On the other hand, if the density of the external medium is increased, the protoplast will be buoyant, creating compression between the extracellular matrix and plasma membrane at the cell-top and a tension between these two at the cell-bottom. This condition will ultimately lead to the reversal of the normal graviresponse (polar ratio < 1). This theory explains the presence of gravitropism in cells lacking statoliths e.g. in certain algae, fungi etc. but, the validity of this model in higher plants and gravitropic mosses is highly questionable as few direct data are available.

Other than these two well-known models, another model called the plasmalemma central control model was proposed by Pickard and Ding, (1993). According to this model, mechanoreceptive ion channels are located in the plasma membrane open in response to friction and flexures in the cell when gravitational forces change. Among

different ions that might be involved here, most attention has been paid to Ca^{+2} (calcium will be discussed in detail later in section 1.1.2.1.1). They speculated that tension in the plasma membrane will open the stretch-activated channels leading to Ca^{+2} release. The calcium ions then can be trapped by putative auxin transport proteins, a kinase (phosphorylating the transport protein) and Calmodulin (Pickard and Ding, 1993). This theory is based on the wall to membrane linkages found in onion epidermal cells (Pontolezica et al., 1993). Mechanoreceptive sensitivity is achieved only if there is a way of transferring shearing stresses from the rigid cell wall to the delicate membrane-filament-channel system, where they can be expressed as strains (Pickard and Ding, 1993). In spite of all these predictions, this theory has not been accepted due to lack of direct evidence for it in gravisensing systems.

1.1.1.3 The Receptors

The receptor is a specialized organ/structure in the cell that interacts with the susceptor in such a way that the physical information of gravity is transduced into a physiological signal (Sack, 1991). The many potential receptors of the signal for higher plants, algae and mosses will be described below, with regard to both plastid based and non-plastid based theories.

1.1.1.3.1 Endoplasmic Reticulum

As mentioned earlier, amyloplast sedimentation is highly likely to be involved in graviperception in the root columella cells. ER has been observed, closely surrounding columella amyloplasts (Sack and Kiss, 1989; Allen et al., unpublished data). It has been proposed that amyloplast sedimentation on distal ER (leading to enrichment in ER) may

function in gravi-sensing (Sievers and Volkmann, 1977). But this theory has been challenged as distal enrichment of ER is not universal (Audus, 1979). Recently, a specialized form of ER called nodal ER has been found in freeze substituted columella cells of tobacco and it may play a role in gravisensing (Zheng and Staehelin, 2001).

Two types of ER have been observed in dark grown protonemata of *Ceratodon*-tubular and cisternal ER (Walker and Sack, 1995). The tubular ER (TER) is distributed evenly throughout all the plastid zones (*Ceratodon* has 5 zones in the amyloplast region of the apical cell) but the distribution of the cisternal ER (CER) is varied among the zones (Walker and Sack, 1995). Both TER and CER are abundant in and near the apex (~35 μm from tip). Longitudinal distribution of the TER changes in the apical cell of gravistimulated protonemata, but the CER remains unchanged (Walker and Sack, 1997). CER is located mostly along the cell periphery in the sedimentation zone and qualitative analysis revealed that plastids sediment on top of the CER in horizontal cells (Walker and Sack, 1997). Although no direct contact between amyloplasts and ER was observed it is possible that ER acts as a gravi-receptor in moss apical cells.

The ER in *Chara* rhizoids is extensive and forms stacks of longitudinal cisternae (Bartnik and Sievers, 1988). The ER distribution is not uniform and it aggregates in the center of the cell tip (Bartnik and Sievers, 1988). The aggregate moves to the bottom (i.e. lower flank) of the cell for sometime during gravistimulation and returns to its central position once vertical growth resumes (Bartnik and Sievers, 1988). Rhizoid treatment with cytochalasin B or phalloidin results in statolith sedimentation and a disappearance of the ER aggregate (Bartnik and Sievers, 1988). So the ER aggregate is thought to be actin dependent and may play an important role in gravity induced growth.

1.1.1.3.2 The Cytoskeleton

All eukaryotic cells have a cytoplasmic network of filamentous proteins and their associated proteins collectively termed the cytoskeleton in their cytoplasm. The cytoskeleton is composed of microtubules, actin filaments (F-actin), intermediate filaments and their associated proteins (Ingber, 1993). In plants, the cytoskeleton has many important roles including cell signaling (Volkmann and Baluška 1999, Nick 1999), tip growth and polarity (Bibikova et al., 1999, Hepler et al., 2001) and perception of environmental stimuli like cold, touch, gravity and light etc (Orvar et al., 2000). Plant gravitropism is a complex process comprised of several well organized, coordinated phases and the cytoskeleton is thought to be an essential component in this pathway.

1.1.1.3.2.1 Role of actin in gravisensing and signaling

The involvement of actin in gravisensing is well documented in the green alga *Chara*. Both positively gravitropic rhizoids and negatively gravitropic protonemata show a complex actin cytoskeleton that plays a role in tip growth as well as in gravisensing by regulating the position and sedimentation of statoliths (Braun and Wasteneys, 1998; Braun et al., 2002). In the rhizoids, the actin cytoskeleton forms thick bundles in the basal region, a meshwork of thin filaments in the subapical zone and a prominent spot of converging filaments in the apex (Braun and Wasteneys, 1998; Braun et al., 1999). In vertical rhizoids treated with cytochalasin D, statoliths fall to the physical bottom of the cell (Hejnowicz and Sievers, 1981). Application of Cytochalasin D leads to loss of the well organized regulation of statolith positioning (Buchen et al., 1993).

There are two different models for cytoskeleton based gravisensing: restrained and unrestrained gravisensing (Baluška and Hasenstein, 1997). In the restrained

gravisensing model, organelles are restricted to their position by the cytoskeleton, which in turn are linked to plasma membrane receptors and ion channels, pumps etc. In response to gravity, these restricted organelles (e.g. statoliths or whole protoplast) can sediment on the cytoskeleton generating a tension that may lead to ion channel activation and triggering of downstream events eventually leading to gravity induced growth (Baluška and Hasenstein 1997; Blancaflor, 2002). According to the unrestrained gravisensing model, the cytoskeleton is not robust enough to control the position and mobility of the organelles and so statoliths sediment to the physical bottom of the cell in response to gravity (Baluška and Hasenstein, 1997). Sedimentation of statoliths onto cellular structures like the ER, the cytoskeleton etc. can then activate downstream events (Blancaflor, 2002). Tensegrity architecture i.e. a cell system built on tensional integrity of the cytoskeleton rather than compressional continuity was first proposed by Ingber et al., (1981). Recently Yoder et al (2001) proposed a new tensegrity based model for gravisensing in roots based on quantitative analysis of statolith sedimentation in corn columella cells. According to this model, sedimenting statoliths disrupt the actin based cytoskeletal network and produce a directional signal that activates/inactivates receptors on the plasma membrane (Yoder et al., 2001).

In spite of all these different models, the actual relationships between sedimenting amyloplasts, the cytoskeleton, ion fluxes and gravitropic growth is not clearly understood and the data often are contradictory. (Blancaflor, 2002). Most of the data is based on immunological studies of the cytoskeleton. The unrestrained model was supported by work from Baluška et al, 1997 in which columella cells were seen to lack prominent F-actin bundles in contrast to other plant cell types with many actin arrays. The new

tensegrity model by Yoder et al, 2001 also shows the absence of actin cables in cryofixed/freeze substituted columella cells. The cytoskeletal matrix in these cells only revealed a meshwork of randomly oriented, single, straight actin-like filaments (Yoder et al, 2001). But recently, prominent actin strands/bundles have been shown to be present in root columella cells by confocal microscopy (Collings et al, 2001; Blancaflor, 2002). These contradictory results arose from lack of proper fixation methods (as the dynamic nature of F-actin is very difficult to image in fixed, dead cells). This problem can be solved by transforming cells with a GFP fusion protein that will accurately label actin filaments.

Cytochalasin D has been the most widely used actin disrupting drug. It blocks membrane potential changes and proton effluxes in gravistimulating roots (Sievers et al., 1995; Monshausen et al., 1996). The mode of action of cytochalasin is complex. It disrupts the dynamic processes at barbed ends of AFs in cooperation with actin binding proteins (Ayscough, 1998) that leads to either overstabilized AFs (Williamson, 1978) or aberrant AF arrays. Though the drug is widely used, there are contradictory results regarding the treatments. Cytochalasin treatment delayed the onset of curvature in *Lepidium* and Cress roots (Wendt et al., 1987; Monshausen et al., 1996) but had no significant effect on curvature in rice, maize and cress roots (Staves et al., 1997b). Latrunculin B a newer actin disrupting drug (Spector et al, 1983) has been more recently used to investigate the role of actin in gravitropism. It has a straight mode of action where it binds to G-actin, forming G-actin latrunculin complexes that cannot polymerize (Coué et al, 1987; Baluška et al, 2001b). Latrunculin B promotes gravitropism in floral stems and hypocotyls of *Arabidopsis* (Yamamoto and Kiss, 2002) and this effect may be due to

a greater ease of plastid movement following disruption of the F-actin network (Yamamoto and Kiss, 2002). Although this supports the theory that statoliths are constrained by the F-actin network, another recent result with cytochalasin D treatment contradicts the hypothesis. Application of cytochalasin D in lentil columella cells leads to a decrease in amyloplast displacement velocity (Driss-Ecole et al., 2000). So although the results with actin disrupting drugs are interesting they further complicate the interpretation.

Localization of myosin-related proteins on the surface of sedimenting amyloplasts in cress root statocytes (Wunsch and Volkmann, 1993), maize root caps (Baluška and Hasenstein, 1997) and of profilin (Staiger et al, 1997), the actin binding protein in alfalfa statocytes and maize root apices (Gibbons and Staiger, 2000) suggest that a dynamic actin cytoskeleton maybe responsible for statolith movements.

Recent genetic studies also support the involvement of the cytoskeleton in the early gravisignaling events. The altered response to gravity (arg1) mutant of Arabidopsis encodes a 45kDa DnaJ-like protein whose C-terminal domain contains motifs with sequence homologous to cytoskeleton –interacting proteins (Sedbrook et al., 1999). This means that ARG1 interacts with the cytoskeleton and may help in transmission of gravity signals to the receptors located at the plasma-membrane or ER (Sedbrook et al., 1999)

1.1.1.3.2.2 The Role of microtubules in gravisensing and signaling

Like actin, the involvement of microtubules in gravity sensing and signaling is unclear (Blancaflor, 2002). MTs are either cortical or endoplasmic in nature (Alberts et al., 1989). Although MT disruption with oryzalin affects amyloplast sedimentation in roots (Baluška et al., 1997), it does not affect root bending (Baluška et al, 1996;

Hasenstein et al, 1999). So there maybe a different role for MTs in root gravitropism. In *Chara* rhizoids, the MT inhibitor colcemid, prevents the gravitropic response (Friedrich and Hertel, 1973). Application of colchicine induces MT disassembly and leads to fattening of cylindrical *Chara* internodal cells to spheres or bulges (Green, 1962). But in contrast to earlier observations oryzalin application does not prevent gravitropism in *Chara* rhizoids (Braun and Sievers, 1993). Again, in the rhizoids and protonemata of the algae *Chara*, exclusion of MTs and convergence of actin filaments in the apex suggests an actin-mediated graviresponse system (Braun and Wasteneys, 1998).

The moss life cycle alternates between a haploid gametophyte and a diploid sporophyte. Diploid spores germinate to produce filamentous protonema (Cove et al., 1997). Protonemal stage is comprised of chloronemal and caulonemal filaments. While caulonema exhibit only axially running MTs, chloronema contain axial, transverse and obliquely arranged MTs (Doonan and Cove, 1985).

Moss MTs were first observed by immunofluorescence methods on moss protoplasts (Powell et al., 1980) and later it has been shown that distribution of MTs is very similar in the various moss species (Doonan and Cove, 1985; Schwuchow et al., 1990). The apical cells of moss protonemata contain MT strands/cables that lie axially and also form a focus at the tip (Doonan and Cove, 1985; Wacker et al., 1988; Schwuchow et al., 1990).

Microtubule rearrangements in fixed moss cells reveal that the graviresponse system involves MT rather than actin. The plastid sedimentation in the apical cell of *Ceratodon* is unusual i.e. only some amyloplasts sediment and they don't fall completely to the new cell bottom (Schwuchow and Sack, 1993). Application of the MT inhibitor

amiprophos-methyl (APM) for 30-60 min lead to increased plastid sedimentation compared to that found in untreated cells (Schwuchow and Sack, 1994). Longer application resulted in more dramatic sedimentation and in some cases almost all plastids sedimented to the lower flank of the cell.

The apical cell of negatively gravitropic protonemata of *Ceratodon* showed a higher density of MTs in the lower flank in comparison to the upper flank when gravistimulated for more than 20 min (Schwuchow et al, 1990). The enrichment of MTs was seen proximal to the sedimenting amyloplasts and near the tip region that elongates to produce curvature (Schwuchow et al, 1990). Application of MT inhibitors like APM and oryzalin lead to disruption of gravitropism and the zonation of the tip cell (Schwuchow et al, 1990).

Application of oryzalin to the protonemata of *Funaria* leads to disruption of zonation in the tip cell and changes the plastid position (Wacker et al, 1988). I used oryzalin in *P. patens* to find out the effect of MT disruption on gravitropism. In this thesis, I also demonstrate the involvement of MTs in early phases of gravitropism in the moss *P. patens*.

1.1.1.3.3 The Plasma Membrane

The plasma membrane is another organelle that could serve as a potential receptor for graviperception. It can contain mechanosensitive channels that could be activated by tension changes (Pontoleizica et al., 1993) occurring directly as amyloplast/whole protoplast sediment or indirectly through stress on the cytoskeleton-plasma membrane interconnections.

1.1.2 Signal Transduction

Signal Transduction is the conversion of a signal (ionic/mechanical/thermal) from one form to another. This signal then gets transmitted to the targeted organ. The order of signaling events occurring after reception are unclear, although some of the players have been identified (Blancaflor, 2002). Below are descriptions of some of the components of signal transduction. These components may act alone or in co-ordination with signaling elements from other stimuli such as touch or light that also induce differential growth.

1.1.2.1 Ions

1.1.2.1.1 Calcium

The cytosolic free Ca^{2+} in the cell remains under strict biochemical and physiological controls (Alberts et al., 1989) and its concentration can be shown to increase following a variety of stimuli. Changes in the level of calcium ions can be used as 'secondary messengers' in plant cells in response to various environmental stimuli. This feature has made Ca^{2+} an attractive candidate as a signaling molecule during the early events of gravitropism (Bush, 1995; Sanders et al., 1999; Fasano et al., 2002). But there are contradictory results regarding direct measurements of cytosolic Ca^{2+} changes. Maize coleoptiles showed an increase in intracellular Ca^{2+} levels within 10 min of gravistimulation (Gehring et al., 1990). In contrast, a very careful investigation using *Arabidopsis* roots showed no change in cytosolic Ca^{2+} levels after gravistimulation, but changes in cytosolic Ca^{+2} after touch stimulation (Leagué et al., 1997). Recently, Plieth and Trewavas, 2002 observed transient changes in cytosolic Ca^{2+} in aequorin (Ca^{2+}

sensing protein) transformed intact *Arabidopsis* seedlings. Most of the data regarding the involvement of changes in Ca^{2+} levels with gravitropism are indirect and involved studies using calcium inhibitors. Application of calcium chelators like EGTA, EDTA etc. to maize roots arrested gravitropic curvature and addition of calcium restored the response (Björkman and Cleland, 1991; Lee et al., 1983). Calcium chelators also block gravity induced auxin redistribution (Young and Evans, 1994). On the other hand, auxin transport inhibitors block cap-based polar Ca^{2+} transport in maize and pea roots (Lee et al., 1984). So there is a relationship between Ca^{2+} and auxin distribution. Calmodulin, a Ca^{2+}-dependent regulatory protein acts as a plant calcium monitor or modulator and is involved in gravitropic sensing and signal transduction. Calmodulin levels in the root apex are elevated in response to gravity (Sinclair et al., 1996). Application of calmodulin antagonists delayed graviresponse in root tips of maize but the root growth was unhampered (Stinemetz et al., 1992).

More evidence regarding the involvement of Ca^{2+} in gravisignaling is based on the studies with the Ca^{2+} related second messenger, inositol-1, 4, 5-triphosphate (IP_3). IP_3 mobilizes Ca^{2+} and helps in its release from intercellular reservoirs like ER, vacuole etc. to trigger subsequent calcium dependent signal transduction pathways (Munnik et al., 1998; Kamada and Muto, 1991; Tucker and Boss, 1996). Recently, a rise in IP_3 level has been observed in oat shoot pulvini within 15 sec of gravistimulation (Perera et al., 1999; Perera et al., 2001). Although, the rise in level was equal in both upper and lower flanks initially, it tripled in the lower flank between 10 and 30 min of gravistimulation (Perera et al., 2001).

It is not clear what the role of Ca^{2+} is during moss gravitropism. Recently, Allen

et al., 2003 observed a Ca^{2+} influx in the apical dome of growing caulonemal filaments of *P. patens*. When these filaments were gravistimulated, the Ca^{2+} influx was not restricted to the apex but extended down the flank for at least 60 μm, where the influx was highest (Allen et al, 2003). So, a differential activation of Ca^{2+} activating channels in the plasma membrane is established following gravistimulation (Fig 4.1 taken from Allen et al., 2003).

1.1.2.1.2 pH

A number of studies have been conducted to measure extra and intracellular pH in response to gravistimulation and comparing the measurements to normal behavior. Proton efflux has been observed in the upper flank of root caps using the vibrating wire probe (Collings et al., 1992). Scott and Allen, (1999) showed that there is a change in cytoplasmic pH in the columella cells of *Arabidopsis* roots that have been shown to be most effective in sensing gravity (Blancaflor et al., 1999). Root cap pH changes are necessary for gravitropism in *Arabidopsis* root (Fasano et al., 2001).

1.1.2.2 The cytoskeleton

The properties and distribution of the cytoskeleton can also be altered by ionic concentration changes. Grabski et al., 1994 used a cell optical displacement assay (CODA: a focused laser beam optically traps and displace transvacuolar and cortical strands through a defined distance within the cell) to quantitatively measure the tension of the cytoskeleton following changes of cytoplasmic pH. An increase in pH caused an increase in cytoskeleton tension while a decrease in pH lead to a decrease in filament tension in soybean and carrot cells (Grabski et al., 1994). Ions and cytoskeleton may

work together to transmit a signal as follows. The decrease of pH in a cell can result in a decrease in actin filament / MT tension leading to sedimentation of heavy cellular components like amyloplasts on to the cell bottom. This in turn could activate putative mechanical or stretch induced ion channels. On the other hand an increase in tension can cause strain on the attached membrane and lead to opening of ionic channels. Also, Ca^{2+} /calmodulin may interact with MTs via MAPs (Microtubule Associated Proteins) (Cyr, 1991) and MT disruption opens Ca^{2+} channels in *Arabidopsis* and carrot cells (Thion et al., 1996, 1998). It is likely that a change in calcium concentration can alter the cytoskeletal distribution of either actin filaments or microtubules such that it induces a biochemical signal that gets transmitted to the target organ.

1.1.3 Response

The response phase of gravitropism involves differential growth of the targeted organ that eventually leads to the redirection of the organ in response to gravity. In roots, growth occurs due to elongation of cells in the upper side of the root. In mosses the apical cell responds to gravity by redirecting its growth away from the gravity vector.

1.1.3.1 The Cytoskeleton

1.1.3.1.1 Actin

Actin may be a regulator of cell elongation (Baluška et al., 2001a; Waller et al., 2000). Elongating plant cells have two types of actin filaments: thick subcortical longitudinal actin bundles and fine cortical transverse actin arrays (Collings et al., 1998; Blancaflor 2000; Collings et al., 2001; Kost et al., 2000). Actin can regulate cell

elongation by mechanically constraining it, by influencing microtubule alignment, or by controlling vesicle transport to active cell wall growth areas (Waller et al., 2000; Baluška et al., 2001b). Presence of severely disrupted actin bundles in the transition zone of the maize *lilliputian* (lacks cell elongation zone) mutant (Baluška et al, 2001a) and generation of dwarf *Arabidopsis* and rye plants after application of the actin inhibitor Latrunculin B (Baluška et al., 2001b) implies that actin may be responsible for normal cell elongation. In spite of these correlations between actin structure and elongation of growth, graviresponding maize roots or pulvini do not reveal any organizational differences of their longitudinal actin bundles between the lower and upper flanks (Blancaflor and Hasenstein, 1997; Collings et al, 1998). But actin disruption in maize root cap leads to enhanced gravitropism (Hou et al., 2003). So actin may act as a regulator of the timing and duration of a signal originating from the root cap that allows the root to resume normal vertical growth (Hou et al., 2003). The fine cortical actin arrays can also play a role in plant morphogenesis through an actin-microtubule feedback system (Collings and Allen, 2000). In developing roots, cortical actin undergoes reorientation similar to microtubule reorientation and it may be possible that cortical actin also modifies during gravity induced growth (Blancaflor, 2000, 2002). But it does not do so in pulvini, where the larger actin filaments appeared to unchanged in orientation during gravistimulation. (Collings et al., 1998).

The dynamic nature of the actin cytoskeleton is due to a wide variety of actin binding proteins (ABPs) (Gibbon, 2001). There are different classes of ABPs: profilins, cofilins, fimbrins, villins, myosins etc. Among these profilins and cofilins are best characterized in plants (Staiger et al., 1997; McCurdy et al., 2001) and play a role in

modulating cell expansion during differential growth (Blancaflor, 2002). Both profilin and cofilin interact with phosphoinositide lipids and respond to changes in cytosolic Ca^{+2} and pH (Gibbon, 2001).

1.1.3.1.2 Microtubules

The involvement of MTs in gravity induced differential growth is based on the parallel alignment of cortical MTs and the newly deposited cellulose microfibrils (Giddings and Staehelin, 1988, 1991). The deposition of microfibrils in the parallel direction restricts the growth (i.e. cell elongation) to a direction perpendicular to its orientation (Green, 1980). Most studies regarding the role of MTs in gravity induced growth have been done in growing roots, pulvini (Collings, 1998) and coleoptiles. Cortical microtubules exhibit a variable but mostly transverse orientation in the meristematic zone that changes to strict transverse MTs in the elongation zone (Barlow and Baluška, 2000). As the cell matures and growth rate decreases, the transverse alignment of MTs changes to a longitudinal pattern (Barlow and Baluška, 2000). Cortical MTs receive directional cues from the environment and transduce this information to different cell developmental processes (Fischer and Schopfer, 1988). In gravitropic roots, the MT orientation changes from transverse to longitudinal in the slower growing lower flank (Blancaflor and Hasenstein, 1993, 1995) while in the lower flank of faster growing graviresponding shoots the orientation changes from longitudinal to transverse (Nick et al., 1990; Himmelspach et al., 1999). So maybe MTs are regulating the gravitropic growth by controlling the directional deposition of cellulose microfibrils (Blancaflor and Hasenstein, 1993).

But there are many counterarguments to this assumption. Firstly, in roots,

reorientation of MTs during gravitropism occurs after the growth curvature has started (Blancaflor and Hasenstein, 1995). Again, plant roots do respond to MT depolymerizing drugs eg, oryzalin etc but that does not prevent gravitropic curvature (Baluška and Hasenstein, 1997). Although application of MT disrupting drugs to maize and rice coleoptiles lead to a reduction in gravitropic curvature (Nick et al., 1991), further investigation of maize pulvini revealed no change in MT organization between upper and lower flanks during gravistimulation (Collings et al., 1998). Lastly, Bichet et al., 2001 demonstrated that *Arabidopsis* BOTERO I mutants with disorganized cortical MTs in the elongation zone can respond to gravity.

In spite of all these studies, some recent findings have again bolstered the fact that MTs play an important role in the plant graviresponse phase. Himmelspach et al., (1999) observed gravity induced reorientation of cortical MTs in tubulin injected live epidermal cells of maize coleoptiles under a confocal laser scanning microscope. A recent study by Himmelspach and Nick, 2001 showed that in maize coleoptiles, gravity induced MT reorientation (30 min in lower flank) preceded gravitropic curvature and growth.

In mosses, MTs are important for the differential growth of the caulonemal apical cell in response to gravity. The moss apical cell has longitudinal arrays of microtubules that extend to the apical dome and converge to form a foci (Doonan and Cove, 1985). Application of oryzalin and APM disrupted MTs, normal tip growth, plastid zonation and gravitropism in the moss *Ceratodon* (Schwuchow et al., 1990). So, MTs serve as an attractive candidate for the gravity induced growth response in some plant graviresponse systems.

1.2 Tip Growth

The gravitropic response in *Physcomitrella patens* occurs as stated previously as a differential elongation of the two sides of the apical or tip filament cell. Below is a discussion of what is known about tip growth in plants.

Plant cell growth exhibits two modes: diffused growth and tip growth. Tip growth is a common form of cell expansion where the synthesis and expansion of cell wall seems to be restricted to the hemispherical apical dome (Doonan et al., 1988; Bibikova et al., 1997). Most of the apically growing plant cells exhibit directional expansion or tip growth. Some of the common tip growing cells are: root hairs, rhizoids, moss protonemata, pollen tubes etc. Ultrastructural analysis of tip growing cells reveals highly organized and polarized cytoplasmic contents (Rosen et al., 1964; Emons 1987). The tip predominantly contains secretory vesicles (Rosen et al., 1964), actin filaments and microtubules (Lloyd and Wells, 1985). The directional expansion requires precise transport and delivery of growth materials (Schnepf, 1986) to the tip. The Golgi vesicles inside the apical dome provides membranes (Picton and Steer, 1983), cell wall matrix precursors (Reiss et al., 1983), cellulose-synthesizing complexes, ion pumps and channels (Brawley and Robinson, 1985) required for cellular extension through polarized exocytosis. Drug inhibition studies indicate that both microtubules and microfilaments (Seagull and Heath, 1980; Picton and Steer, 1982) may be necessary for the transport of these materials to the tip. Calcium ions also play an important role in tip growing cells. The following study will mainly focus on the role of cytoskeleton and calcium in the regulation of growth in tip growing cells.

1.2.1 Calcium and Tip growth

Tip growth has been most extensively studied in root hairs and pollen tubes. A calcium influx at the tip is an essential requirement for growth in both. The apical areas of growing cells exhibit a gradient of high concentration of free cytosolic calcium (Bibikova et al., 1997; Felle and Hepler, 1997; Wymer et al., 1997).The steepness of the tip-focused gradient and growth rate of the cells are directly proportional to each other. This local increase in calcium concentration disappears when the growth terminates (Wymer et al., 1997; de Ruijter et al., 1998). Application of calcium ionophores and channel blockers lead to growth inhibition in root hairs and pollen tubes (Herth et al., 1990; Wymer et al., 1997).

The increase in calcium concentration at the apex is due to a calcium influx (Herrmann and Felle, 1995; Jones et al., 1995; Malho et al., 1994). Electrophysiological studies using the ion selective biocurrents probe shows that the calcium influx is higher at the tip than at the sides or base of the growing cell (Schiefelbein et al., 1992; Herrmann and Felle 1995; Jones et al., 1995; Pierson et al., 1994). When growth ceases the calcium channels at the apex are thought to be closed. Vesicle fusion is enhanced in the presence of calcium concentration higher than normal cytosolic level (Zorec and Tester, 1992; Wollheim and Lang, 1994). The concentration of calcium at the cell apex determines the direction in which the secretory vesicles deposit materials (Gilroy and Jones, 2000). In mosses, growing caulonemal filaments of *P. patens* also have a calcium influx at the apical dome (Allen et al., 2003).

In root hairs, MT disruption produces a new tip with a tip focused calcium gradient (Bibikova et al., 1999). Again, application of an artificial calcium gradient (by

localized photoactivation of a caged calcium ionophore) across root hair tip results in reorientation of root hairs toward the new calcium gradient (Bibikova et al., 1997). UV photoactivation of caged calcium in pollen tubes also results in growth towards the increased calcium level (Malho and Trewavas, 1996). An increase in calcium levels leads to an increase in calcium dependent protein kinase levels (Moutinho et al., 1998). This increase in protein level may then regulate Ca^{+2} channel activity (Moutinho et al., 1998) that can lead to changes in the actin cytoskeleton (Kohno and Shimmen, 1987).

1.2.2 Cytoskeleton and Tip growth

1.2.2.1 Actin

In plants the actin cytoskeleton is essential for the elongation of tip growing cells (Cai et al., 1997; Kropf et al., 1998). Application of actin disrupting drugs to apically growing plant cells immediately inhibits their growth (Cai et al., 1997; Kropf et al., 1998). Cytochalasin-induced disruption of actin inhibits vesicle trafficking and exocytosis and leads to the disappearance of apical ER aggregates (Bartnik and Sievers., 1988; Bartnik et al., 1990). All these events eventually result in termination of tip growth (Hejnowicz and Sievers., 1981; Braun and Sievers., 1993). Rac/ Rho/Cdc42-related small GTPases play an important role in tip growth and also regulate actin dynamics (Smith, 2003). In pollen tubes, constitutive expression of Rac causes a change in actin orientation from longitudinal to spiral and dominant negative form of Rac causes a reduction of actin bundling and growth inhibition (Kost et al., 1999). So actin architecture is necessary for directional excocytosis and tip growth.

1.2.2.2 Microtubules

Although MTs are essential component of the cytoskeleton, their involvement in tip growth varies from system to system. MTs are not essential for pollen tube growth and tip extension. When MTs are disrupted, the cytoplasmic organization gets altered but cell elongation and overall cell morphology remains unchanged (Kost et al., 1999; Taylor and Hepler, 1997; Derksen et al., 1995).

In contrast, MTs regulate tip growth in root hairs and moss protonemata. In root hairs, the cortical MTs run parallel to the long axis of the cell and extend to the apex. Bibikova et al., 1999 demonstrated that both stabilization and depolymerization of MTs in *Arabidopsis* root hairs causes a loss of directionality and induces multiple growth points at the apex. Again, the inhibition of cytoplasmic streaming due to actin depolymerization in *Hydrocharis* root hairs is reversible in presence of MTs (Tominaga et al., 1997). This indicates that actin organization in root hairs may be regulated by MTs (Kost et al., 1999; Collings and Allen, 2000).

In mosses, the polarity of the protonemal tip cell is MT regulated (Doonan et al., 1988). The moss apical cell has longitudinal arrays of microtubules that extend to the apical dome and converge to form a foci (Doonan et al., 1985). Microtubule-perturbing drugs can cause dramatic abnormalities in protonemal growth. MT disruption in moss caulonemal cells results in a new swollen growing point (Schnepf, 1983; Doonan et al., 1988; Wacker et al., 1988; Schwuchow et al., 1990; Meske et al., 1996) but MT stabilization lead to a reduction in the swelling and causes repolarization of the outgrowths (Doonan et al., 1988). So, normal MT distribution is essential for protonemal tip growth.

2 MATERIAL AND METHODS

2.1 Culture of *Physcomitrella patens*

Protonemal fragments of *P. patens* were grown for 10-12 days under continuous white light at 23^0C on solid 0.7% Agar with {$PPNH_4$: $CaNO_3.4H_2O$(0.08%), $MgSO_4.7H_2O$(0.025%), $FeSO_4.7H_2O$(0.001%), Microelements, NH_4tartarate(0.05%), Glucose(0.5%)} media, but without glucose (Under Annex A: http://www2.unil.ch/lpc/docs/pdf/Schaefer_Thesis.pdf).The fragments were overlaid with cellophane disks (W.E. Canning, Bristol, UK) After 10-12 days, the protonemal fragments were plated on $PPNH_4$ media (with glucose) and grown in the dark in a vertical orientation (Fig. 1.3) for 6 days. Filaments were embedded in 1.3% low melting agarose (Sigma, Type VII) under green light, placed on a slide in which a hole had been drilled in the center (Fig. 2.1) and a cover glass (# 0) had been placed at the bottom. This formed a chamber. The agarose was supplemented with glucose medium. Plantlets were allowed to recover overnight in the medium and in darkness.

12 hours later, the filaments were gravistimulated (by rotating the slides 90^0 to the vertical) for 15, 30, 60 and 120 min and fixed in the same orientation. Gravistimulation was carried out on 1 plantlet per replicate (each plantlet had about 5-6 filaments and there were 4 replicates/ experiment) and each experiment was repeated 4 times. Microtubules were labeled by indirect immunofluorescence technique. Total number of filaments imaged at each time point was approximately 30.

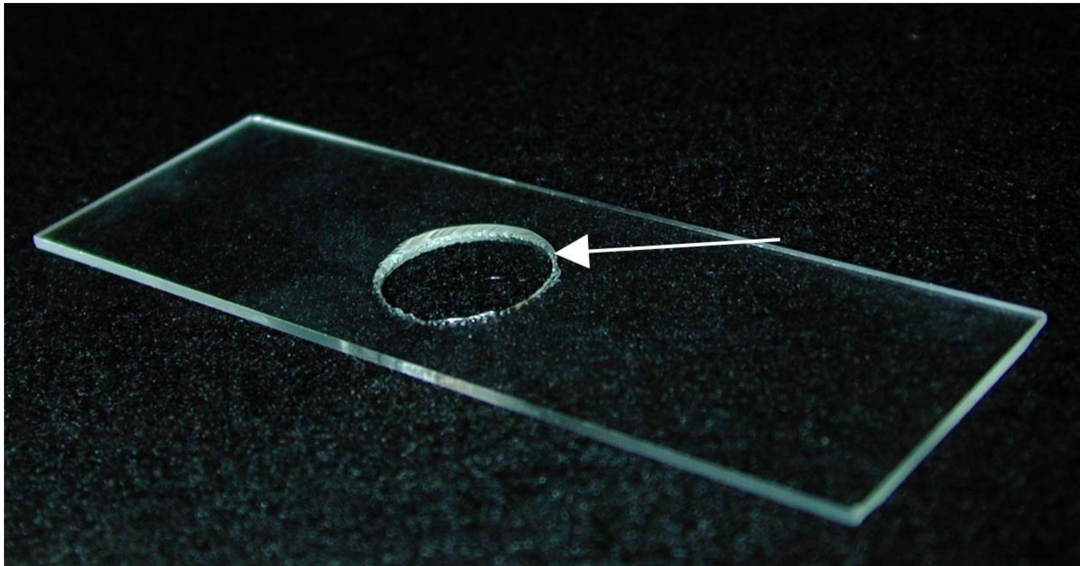

Fig 2.1 Slide with a hole in the center.

A slide with a hole (arrow) in the center was used for all the experiments. A cover glass (# 0) was silicon-greased onto the bottom of the slide forming a small chamber in which small plants with many filaments were placed.

2.2 Indirect immunofluorescence

Filaments were placed for 1.5 hrs. in a fixative containing MTSB (150mM Pipes, 6mM $MgSO_4$,15mM EGTA, pH 6.9), 1% DMSO, 0.01% Triton X-100, 8% Formaldehyde). Filaments were washed 4x in phosphate buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH_2PO_4, 13.69 mM NaCl, 0.81 mM Na_2HPO_4, pH 4), placed in acetone and then kept in the freezer for 10 min. 4 washes of PBS were given after every treatment. Filaments were incubated in blocking buffer (5% BSA, 0.05% Tween-20 in PBS) for 15 min. followed by incubation in monoclonal anti-alpha tubulin (N356; Amersham International plc, Buckinghamshire, England) diluted 1/100 in incubation buffer (1% BSA, 0.05% Tween-20 in PBS). Filaments were then washed and incubated with secondary antibody (Alexa Fluor 488 goat anti-mouse IgG diluted in 1/400 in Incubation buffer, Molecular Probes, Eugene) for 2hrs at 37^0C. After incubation, the filaments were washed and mounted with PBS, slides were covered with No. 0 cover slips and sealed with VALAP (Vaseline, Lanoline, Paraffin: 1:1:1).

2.3 Imaging

Images were taken with a confocal microscope (Leica TCS Nt or TCS SP or LCS; Wetzlar, Germany) with a 40x, N.A. 1.4 oil immersion objective. Optical stacks were recorded using approximately 0.2 – 0.3 μm thick sections and each plane was averaged 4 times. Alexa Flour 488 was excited at 488 nm and emission collected from 500-550 nm. Images were processed using Leica confocal software and Adobe Photoshop 5.0.2. Although the actual fluorescence images look green under the microscope they were given a pseudo color of “glow” for better analyzing power and visual appearance.

2.4 Intensity calculations

Fluorescence intensities were calculated using the Metamorph Analysis System (Universal Imaging). Approximately the same areas from the upper and lower flanks were selected using the region tool and the intensity of those areas was calculated using the 'Region measurement' tool. The intensity data was exported to an excel spreadsheet. The means of upper and lower flank intensities for each time frame were calculated. Next, the ratio of the mean intensities (lower flank/upper flank) was calculated and the data was imported to Microcal Origin software and a graph was constructed.

2.5 Immunolocalization of oryzalin treated filaments

Prior to gravistimulation the filaments were treated with 0.1 μM oryzalin (microtubule depolymerizing drug) for 5 min and then indirect immunolocalization was performed in exactly the same way as above (under indirect immunolocalization).

2.6 Growth Experiments

P. patens was grown under continuous white light in exactly the same way as described in section 2.1. After 10-12 days, the protonemal fragments were placed on $PPNH_4$ medium (0.5% phytagel was substituted for agar in order to obtain a transparent medium) with glucose and grown in the dark for 6 days. After 6 days the filaments were treated with 0.1 μM oryzalin for 5 min under dim green light. After 5 min, oryzalin was washed away by medium (twice) and the filaments were gravistimulated by rotating the Petri plate (the Petri plate was glued to the slide with the usual hole) 90^0 to vertical. At specific time points, the slides with Petri plates were mounted on the rotating stage of an upright microscope (Leica model DMRX-A). The microscope was mounted sideways in

a cradle to give a horizontal light path (Fig 2.2). Images were recorded by using a camera (DAGE- MTI CCD 100, Dage-MTI, Inc., Michigan City, IN) with its IR blocking filter removed to make it sensitive to IR and image analysis was performed with the Adobe Premiere software.

Fig 2.2 A-B Leica Upright Microscope cradled in a sidemount position.

(A) Leica upright microscope (DMRX-A) used for growth studies.
(B) Infrared camera (DAGE- MTI CCD 100) used to capture images.

3 RESULTS

Microtubule distribution in dark-grown normal filaments

Caulonemal filaments of *P. patens* are negatively gravitropic (Jenkins et al., 1986). The microtubules in the apical cell of such dark-grown filaments were imaged with a confocal laser scanning microscope. Fig. 3.1a shows one typical pattern of MT distribution in normal vertically oriented cells. In 93% of the filaments, the MTs were predominantly distributed in an axial pattern. Fig 3.1b shows a transmitted light image of a vertical filament. In DIC images the apical cell has a plastid free tip followed by an area containing many amyloplasts (arrow). The apical cell is filled with MTs and in some cells the MTs do not form a cap (Fig 3.1a) while in others they merge at the apex to form a dense cap (Fig.3.1c, asterisk). The MTs also wrapped around the amyloplasts, which are visible as darker areas in the fluorescent images (Fig 3.1a, arrow). The MTs formed a dense network throughout the anterior part of the apical cell. Both endoplasmic and cortical MTs are visible when the collected confocal sections are reconstructed into 3D images (physcovetical.avi). The fluorescence intensity level of MTs was very similar in all sections imaged throughout the cells indicating an equal distribution of MTs in 3-dimensions. Intensity analysis by Metamorph software revealed an intensity ratio of 0.99 (I_L/I_U) with std. error of 0.04 agreeing with the qualitative 3D observations.

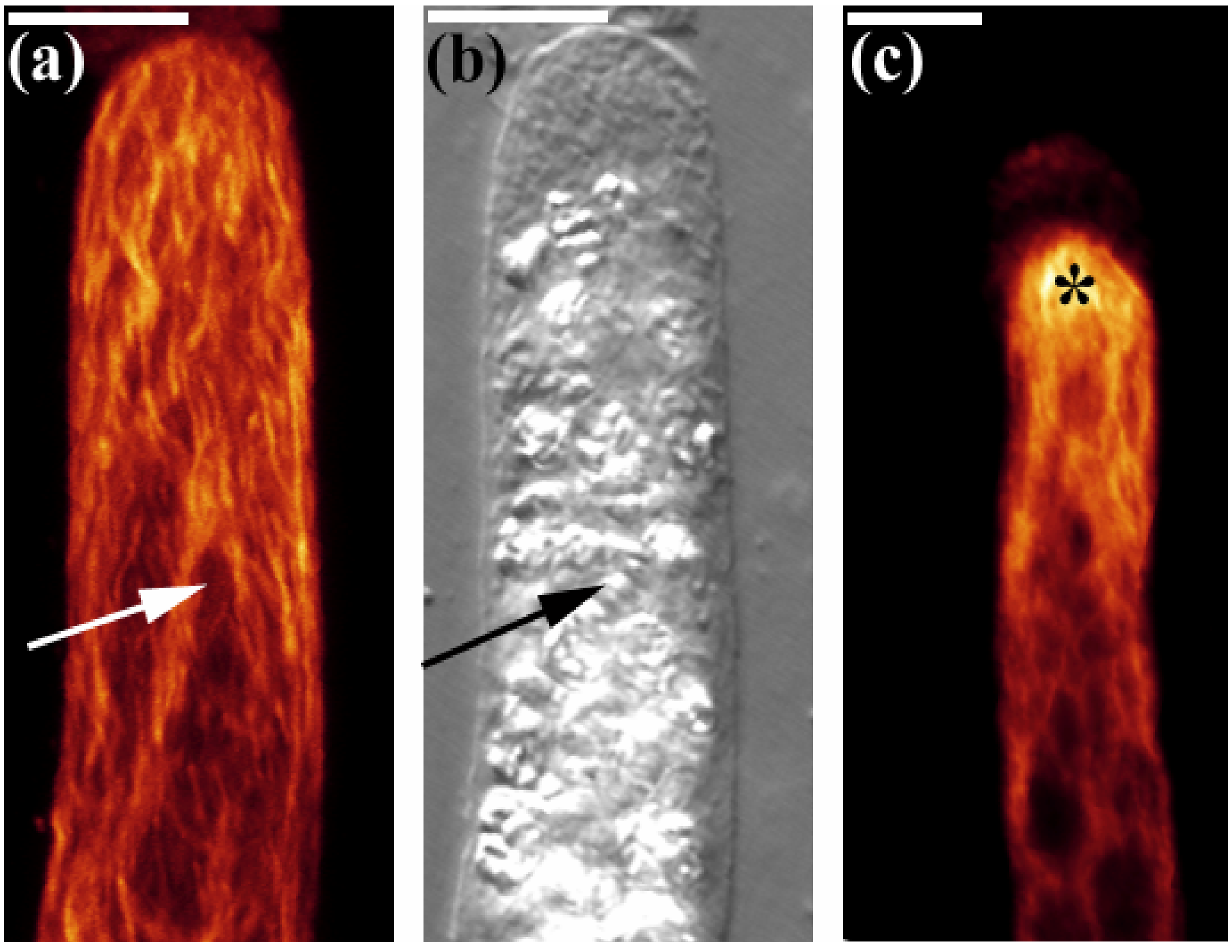

Fig 3.1 a-c Microtubule distribution in vertically oriented filaments.

Full stack projections of fluorescence and transmitted light images of upright filaments as observed through a confocal laser scanning microscope (CLSM). Fluorescently labeled filaments display a net axial distribution of MTs with either no cap (a) or a dense apical cap (c, *). The DIC image (b) is the same filament as seen in (a). Each amyloplast (arrow) is located in a distinct area and is clearly visible in the DIC image (b) Bar = 10 μm.

No significant changes are seen in overall MT distribution 15 min after a change in the gravity vector

Caulonemal filaments were gravistimulated by changing their orientation (i.e. 90^0 to vertical). The distribution of MTs in these filaments (~84%) deviated only slightly from that of the non-gravistimulated ones. MTs were very dense and formed an apical cap in most filaments (Fig.3.2a). The level of fluorescent intensities between the upper and lower flanks revealed no significant difference.

Significant changes in tubulin distribution are observed 30 min after gravistimulation starts

About 76% of the filaments gravistimulated for 30 min were enriched in tubulin in the lower flank of the cells just below the amyloplasts (approx 20 μm from tip) as compared to the upper flank (Fig. 3.2c, arrow). The enrichment in tubulin was observed as a higher intensity in the lower flank occurring behind the amyloplast free zone of the tip i.e. in the plastid zone. The axial MTs are still present and form an apical cap. MT strands are visible throughout the cell. The amyloplasts are visible in Fig. 3.2b (arrows) and as corresponding dark areas without fluorescence in Fig. 3.2c (arrows). The accumulation of tubulin is visible clearly in a 3D image (physco30mingravistimulated.avi). Analysis with Metamorph revealed and fluorescence intensity ratio of 1.4 with std. error of 0.02 between the upper and lower flank.

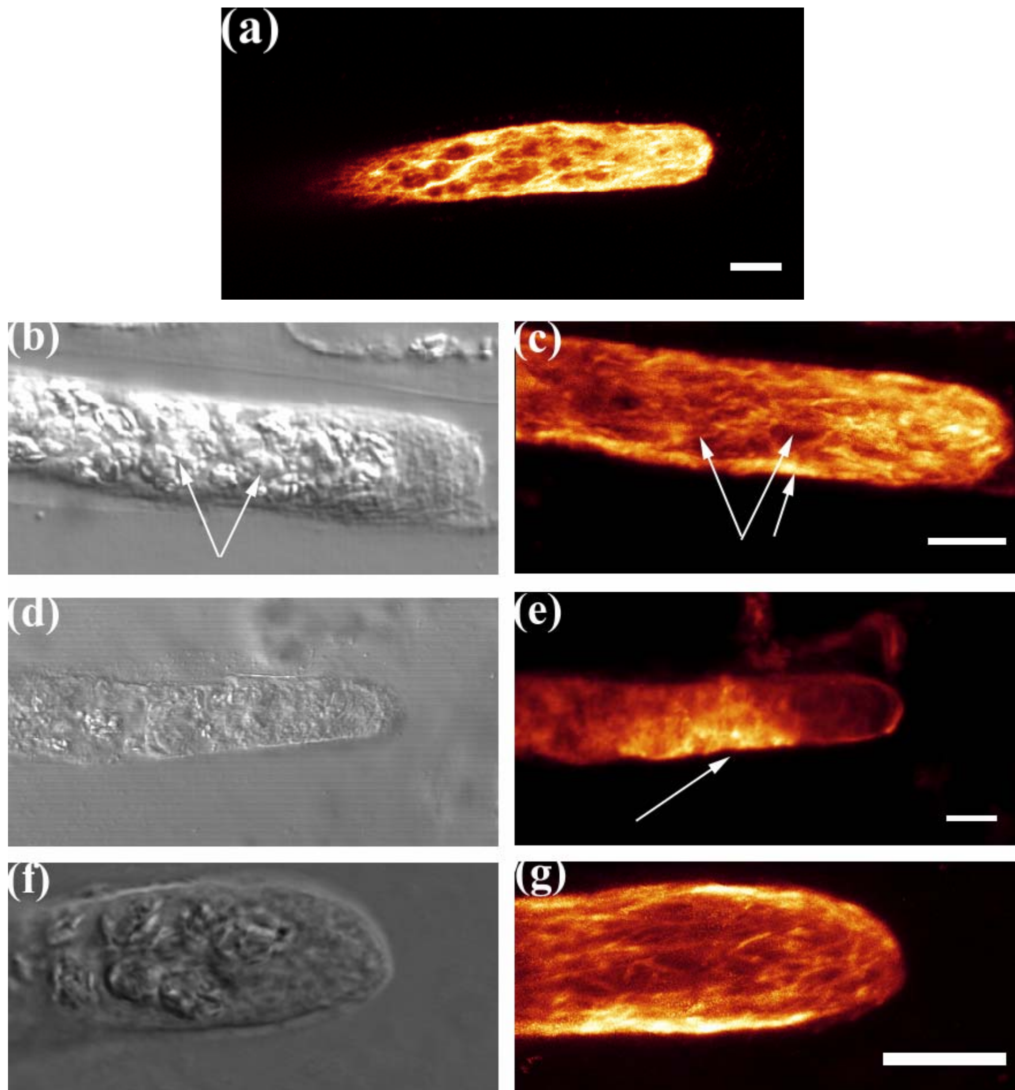

Fig 3.2 a-g Microtubule distribution in filaments that were gravistimulated for 15, 30, 60 and 120 minutes.

CLSM fluorescence and transmitted light images of gravistimulated filaments taken at different time points. MT distribution is almost the same in both flanks at 15 min (**a**) however the accumulation of tubulin in the lower flank is distinct at 30 min (**c**) and 60 min (**e,** arrow). At 120 min the overall accumulation in the lower flank is more than that of the upper flank (**g**). The transmitted light images (**b**, **d**, **f**) show the plastid free tip zone and amyloplasts that correspond to dark areas in the fluorescent images. Intensity measurements were made on many such fluorescent images. Bar = 10 µm.

60 min after gravistimulation, the greatest accumulation of tubulin occured at the lower flank

When filaments were on their side for 1hr MTs disintegrated, but a very significant enrichment in tubulin fluorescence on the lower flank was observed in 71% of these filaments. The increase occurred in the plastid zone, behind the amyloplast free zone in the tip cell (Fig.3.2e). An arrow indicates the area that had quite intense labeling. Corresponding transmittance light images revealed that all the points of high intensity were in the regions surrounding plastids (fig 3.2d). The preservation of these filaments was difficult, since the MT filaments were not there. The intensity ratio of the fluorescence between the lower and upper flank (I_L/I_U) was 1.7 with a std. error of 0.22.

Tubulin density returned to low levels 2 hr after gravistimulation

Gravistimulation for 2hrs (Fig.3.2g) appeared to increase the tubulin density in the lower flank, but analysis of the intensity levels revealed that the difference in intensity between the upper and lower flank had decreased relative to that found in cells gravistimulated for 1hr treatment. The tubulin intensity is almost similar to that measured at 30 min and the intensity ratio is also similar to the 30 min level (~1.299) with a std. error of 0.015. Strands of MTs are visible and they lie in the axial direction (3.2g).

Graphical representation of intensity ratio

Fig 3.3 clearly demonstrates the intensity differences occurring between the upper and lower flanks at different time points after gravistimulation.

Tubulin changes associated with gravistimulated filaments has been illustrated with a summary cartoon (Fig 3.4) and the total number of filaments showing tubulin changes has been displayed under Table 3.1.

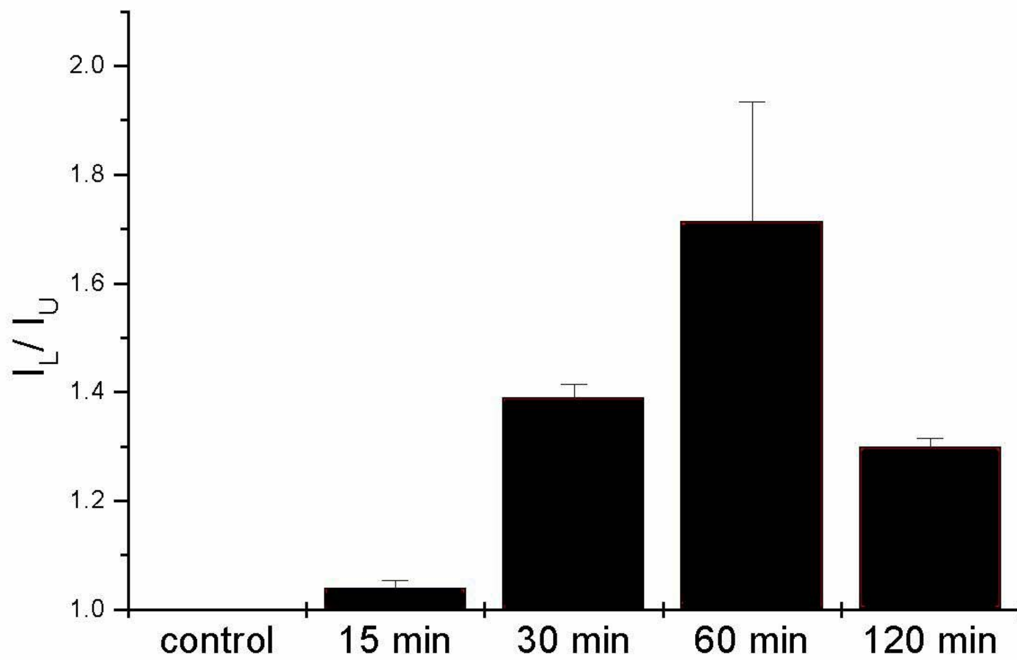

Fig 3.3 Graphical representation of the ratio of fluorescence intensity of the upper and lower areas of gravistimulated filaments.

The intensity ratio (I_L/I_U) quantitatively demonstrates that a significant increase in fluorescence occurs 30 min after gravistimulation with a peak increase after 60 min. The intensity ratio decreases at 120 min to the 30 min level. n = 5 filaments/bar.

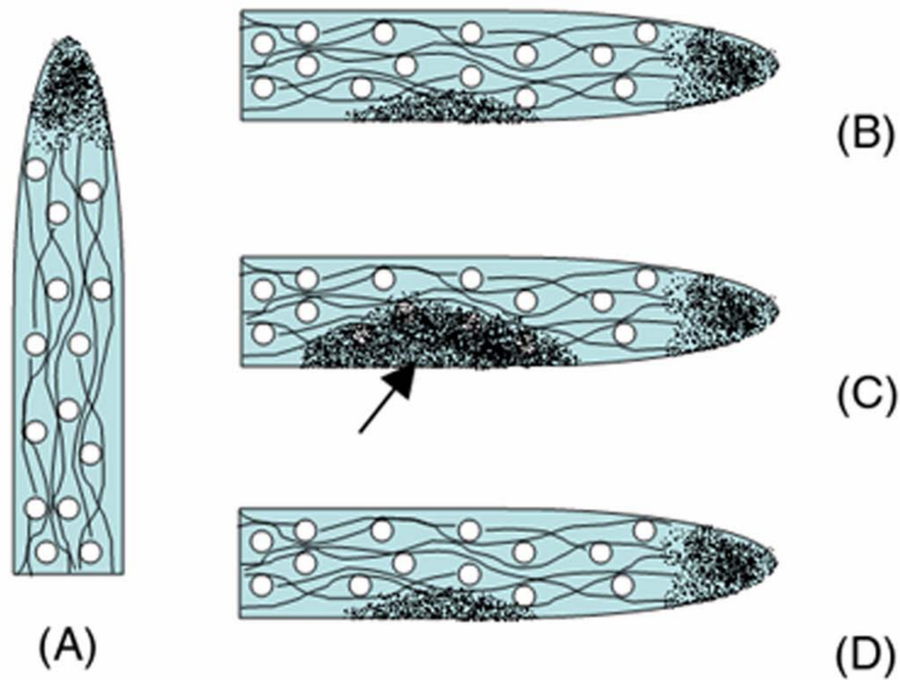

Fig 3.4 Cartoon of MT distribution before and after gravistimulation.

This cartoon illustrates the distribution of tubulin in the lower flank of an apical caulonemal cell before and after gravistimulation. In a non-gravistimulated cell the MTs exhibit axial distribution with the MT distribution almost the same between the two flanks (**A**). The tubulin concentration increases in the lower flank 30 min after gravistimulation (**B**), peaks after 60 min in area of the arrow (**C**) and returns to 30 min level after 120 min (**D**).

Time Points	Numbers	% (Approx)
Control (no cap)	4/29	14
Control (with cap)	23/29	79
15 min	21/25	84
30 min	22/29	76
60 min	20/28	71
120 min	24/30	80

Table 3.1 Total number and percent of cells with or without tubulin distributional changes

In order to determine whether MT redistribution is important for gravistimulation, I treated the caulonemal filaments with oryzalin (0.1 μM) for 5 min prior to gravistimulation and recorded their behavior. Gravistimulation was performed for 30 min, 60 min and 4 hrs. This level of oryzalin is non toxic to the filaments as the filaments continue to grow after an initial arrest. Gravistimulation was performed for 30 min and 60 min as the most noticeable tubulin changes in gravistimulated filaments occurred at these time points.

MT depolymerization visible after oryzalin treatment of both upright and gravistimulated filaments

After treatment with oryzalin both upright and horizontal filaments had depolymerized MTs. Long MT strands were not visible and the entire apical cell was filled with short strands of MT (Fig. 3.5a). Diffuse staining indicated the presence of broken MTs and tubulin dimers. Although both vertical controls and horizontal filaments showed an enhanced fluorescence near the tip, the distinct cap like structure that was present in the untreated filaments was absent here (Fig. 3.5a). Many filaments had a bulge a little behind the tip implying that the bulge had formed following MT depolymerization due to oryzalin application (Fig. 3.5a, arrow).

No major changes happened in MT distribution in oryzalin treated 30 and 60 min gravistimulated filaments

In contrast to the earlier observations, about 98% of 30 min (Fig. 3.5b) and 97% of 60 min (Fig. 3.5c) gravistimulated filaments had an equal distribution of MTs in both the upper and lower flanks. Due to treatment with oryzalin the MTs were depolymerized

and sometimes showed very distinct punctate labeling of MTs (Fig. 3.5 e, f).

Accumulation of MTs in the lower flank of oryzalin treated filaments is delayed, but finally is observed 4 hrs after gravistimulation

About 75 % of oryzalin treated filaments showed a preferential accumulation of MTs in the lower flank in comparison to the upper flank 4hrs after gravistimulation (Fig. 3.5d). The accumulation occurred in approximately the same region as seen in normal gravistimulated filaments earlier. Some filaments also showed reformation of MT strands (Fig. 3.5d).

Total number of filaments showing MT changes is illustrated under Table 3.2.

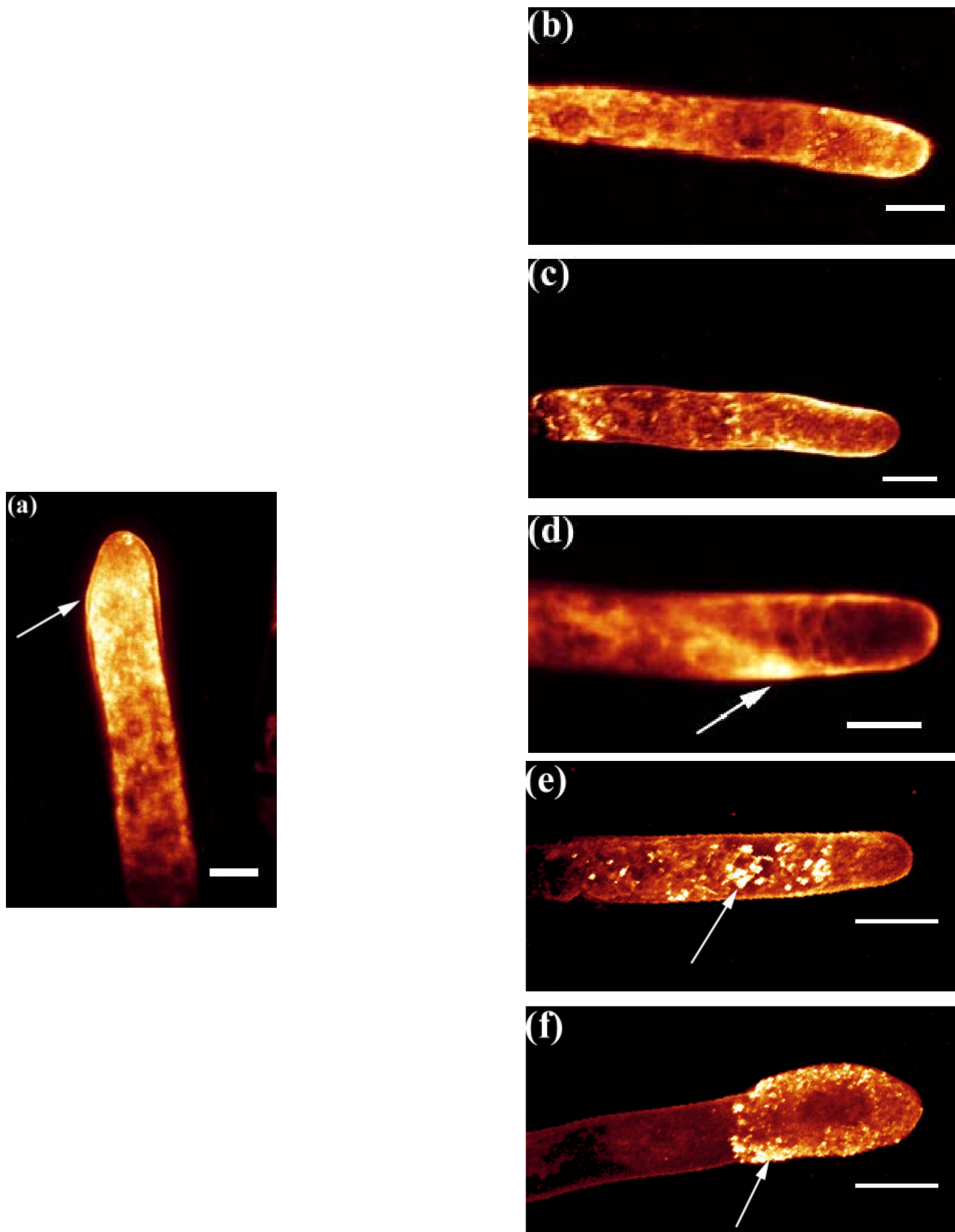

Fig 3.5 a-f. Microtubule distribution in oryzalin treated filaments.

CLSM fluorescence images showing disruption of MTs following oryzalin application in a vertical control (**a**). The distribution of MTs between the upper and lower flank of oryzalin treated filaments is the same after 30 min (**b**) and 60 min (**c**) of gravistimulation. Oryzalin treated filaments show obvious accumulation of MTs in the lower flank after 4hrs of gravistimulation (**d**). Bar = 10 µm. CLSM fluorescence images showing punctuate labeling after oryzalin application (**e, f,** arrows). **f**, is an example where the apex of the filament bulged due to oryzalin application.Bar = 20 µm.
c, **e** & **f** demonstrate the variability in staining and growth patterns.

Time Points	Numbers	% (Approx)
30 min	39/40	98 %
60 min	28/29	97 %
240 min	15/20	75 %

Table 3.2 Total number of filaments showing either no change or changes in tubulin distribution after oryzalin application

Growth Experiment Results

No visible growth changes occur 15 min after oryzalin application

About 99% of the filaments [both gravistimulated and non-gravistimulated (Fig. 3.6a)] show no visible change in growth 15 min after application of oryzalin. The apical cell has a plastid free zone at the tip that is followed by the dense amyloplast zone (Fig 3.6a). The amyloplasts are arranged through the middle of the cell leaving two thin clear stripes of cytoplasm on either side.

Bulge formation starts 75 min after oryzalin application

About 90 % of both vertical controls and gravistimulated filaments show a bulge either at the tip (Fig 3.6b, 3.7b) or a little behind the tip (Fig 3.7a). In some apical cells the amyloplasts move towards the tip of the cell (Fig 3.7b, arrow). While the bulge is formed there is a temporary arrest in tip growth of the apical cell.

Movement of amyloplasts towards the lower flank in gravistimulated filaments

After 105 min approximately 91% of gravistimulated filaments had a bulge with the amyloplasts aggregating and settling to the bottom of the apical cell (Fig 3.7c, 3.7d). After 135 min of oryzalin application 76 % of filaments had a bulge while 24% had branching at the tip. The movement of the amyloplasts towards the lower flank becomes more prominent around this time (Fig 3.7e, 3.7f). The tip growth resumes and in gravistimulated filaments the tip shows prominent negative gravitropism after about 2 hrs 15 min (Fig 3.7e).

Formation of bi and multipolar branches in gravistimulated filaments

165 min after oryzalin application about 60% of gravistimulated filaments show bi or multi-polar branching at the tip (Fig 3.7g, 3.7h). In vertical filaments unbranched

filaments had reinitiated tip growth (Fig 3.6c, 3.6d). The amyloplasts form aggregates and move to each branch tip (Fig 3.7h). After about 240 min (4hrs) growth continues at a regular pace and amyloplasts continue to settle to the lower flank of gravistimulated filaments (Fig 3.7i, 3.7j).

24 hrs after oryzalin application growth continues:

Any one of the branches from bi/multipolar tip may continue to grow while others stop growing. Growth continues in both vertical (3.6e, 3.6f) and in horizontal filaments (Fig 3.7k, 3.7l).

Table 3.3 displays the total number of filaments showing bulges and or branches.

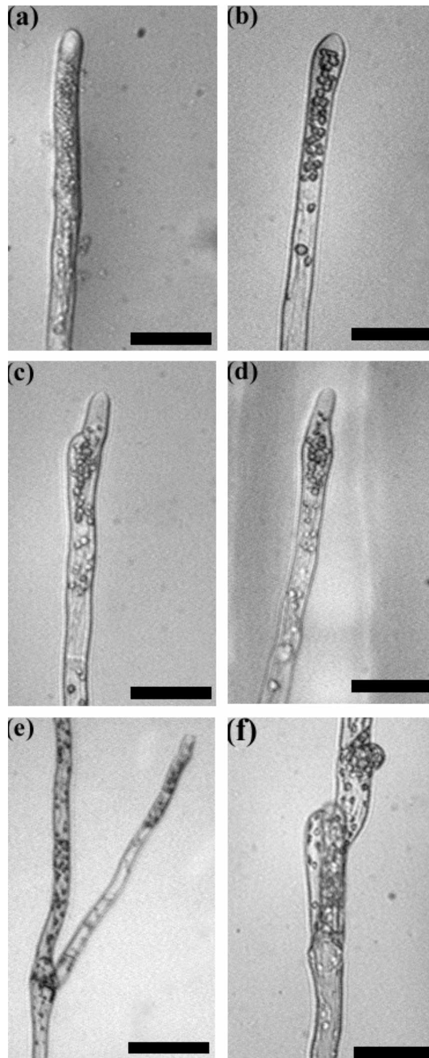

Fig 3.6 a-f. Oryzalin induced growth changes in non-gravistimulated filaments.

The morphology of oryzalin treated vertical filaments look normal at 15 min after application (**a**). The filament tips become bulbous (90 %) 75 min after application (**b**). 27 % tips exhibit branching (**c**) and 73 % reinitiate tip growth (**d**) 165 min after oryzalin application. After 24 hrs (**e**, **f**) the growing filaments respond to gravity from the new growth point. Bar (a-d & f) = 50 μm, e = 100 μm

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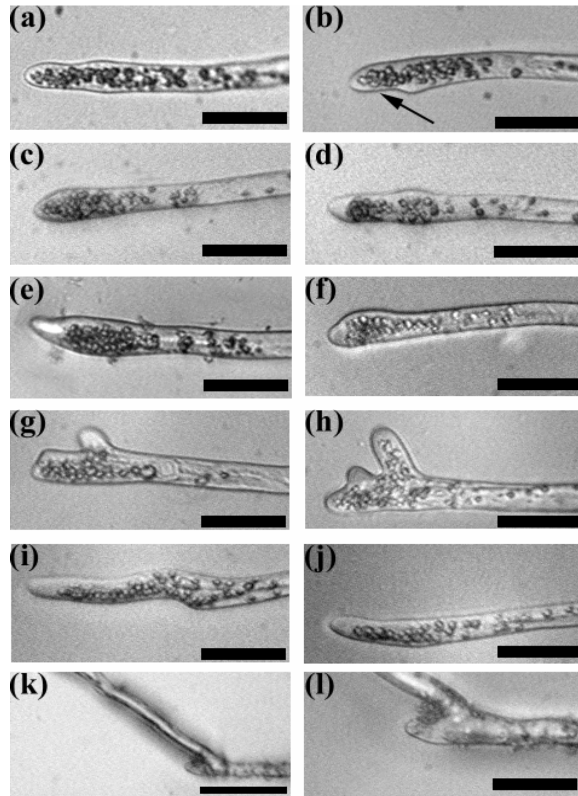

Fig 3.7 a-l. Oryzalin induced growth changes in gravistimulated filaments.

Gravistimulated filaments exhibiting bulges 75 min (**a**, **b**) and 105 min (**c**, **d**) after oryzalin application. The oryzalin treated filament resumed growth and responded to gravity around 135 min after application (**e**, **f**). Amyloplast settling towards lower flank is visible (**d**, **e**, **g**). Both branched filaments (exhibiting either bipolar (**g**) or multipolar (**h**) branching) and un-branched filaments (**i**, **j**) continue to grow. After 24 hrs branched filaments clearly show gravitropism from the new growth point (**k**, **l**). Angle of new growth is often quite characteristic. Bar = a-j & l = 50 μm, k = 100 μm.

Time Points (gravistimulated)	Numbers	% (Approx)
15 min	30/30	99
75 min (Bulge)	28/31	90
105 min (Bulge)	29/32	91
135 min (Branch)	7/29	24
135 min (Bulge)	22/29	76
165 min (Branch)	18/30	60
165 min (Bulge)	11/30	37

Table 3.3 Number of filaments (%) showing bulging and branching over time

4 DISCUSSION

4.1 Tubulin distribution changes occur during gravistimulation

This study demonstrates that early stages of gravity sensing and signal transduction cause a dynamic change in tubulin distribution in the caulonemal apical cell of *P. patens*. The first visible changes appeared in the cells ca. 30 min after the gravity vector was changed. An accumulation of tubulin was seen in the lower flanks of the gravistimulated filaments increasing remarkably around 60 min and then returning at 120 min to levels comparable to those measured at 30 min. The MT changes are illustrated under Fig 4.2. These dynamic microtubule and tubulin movements always occurred in the amyloplast zone about 20 micrometers below the cell apex and leaving the MT distribution in the amyloplast free tip zone unaltered. These data suggest that tubulin changes are associated with gravitropism in *Physcomitrella* and they confirm previous similar studies on another moss, *Ceratodon* as described in the introductory section 1.1.1.3.2.2.

In parallel with earlier studies, I show that MTs in *P. patens* also are on the whole distributed in an axial manner and form a dense cap-like structure at the tip of the cell. Similar to the earlier observations (Doonan and Cove, 1985) I observed that MTs surround the plastids. The ability to make 3D reconstructions from confocal sections allowed visualization of both cortical and endoplasmic MTs and showed that they form an intricate meshwork in the apical cell (physcovertical.avi).

Schwuchow et al., 1990 had shown that MTs are important for the gravitropic

responses of *Ceratodon,* but it was not known if their role in *P. patens* relative to gravitropism was similar or not. In *Ceratodon* the accumulation of MT in the lower flank of the apical cell is proximal to the sedimenting plastid zone (Schwuchow et al., 1990). In this study I report tubulin enrichment in the plastid sedimenting zone of *P. patens*. The tubulin is observed to be accumulating in the anterior part of the amyloplast zone towards the lower flank, and the tubulin intensity varies at different time points. This is in contrast to events in *Ceratodon* where the enrichment intensity does not vary with time.

Unlike *Ceratodon* where plastid sedimentation is rapid (observed within 15 min of gravistimulation, (Walker and Sack, 1990), *P. patens* has a slower and more subtle plastid sedimentation (Schwuchow et al., 1995, video recordings by Allen et al., 2003). In these cells, a thin plastid free strip of cytoplasm is visible in the upper flank of the apical cell about 52 min after gravistimulation (Knight and Cove., 1991; Schwuchow et al., 1995). Based on this study and my data it can be said that the initial tubulin accumulation may precede amyloplast sedimentation in *P. patens*. However, the time of peak tubulin intensity and sedimentation coincides at around 50-60 min after gravistimulation. MTs are thought to be involved in signal perception and transduction in concert with cell wall materials, plasma membrane receptors and MT associated proteins (Kropf et al. 1998; Gundersen and Cook 1999). So MTs are an essential component involved in gravity perception and transmission of signals in *P. patens* and no MTs means no gravisensing as discussed below.

4.2 Effect of Oryzalin

Treatments with oryzalin transiently arrests tip growth with formation of bulges (Fig. 3.6b, Fig 3.7a, 3.7b & 3.7c) and/or branches (Fig. 3.7g & 3.7h) occurring at the tip of the filament. As in *P. patens* normal networks of MTs are essential for tip growth (Doonan et al., 1988), MT disruption may lead to loss of polarity that is displayed as bulging/branching. So it may be assumed that the integrity between the growing tip and network of cytoplasmic MTs is crucial and if the MTs loose their functionality abnormal growth is the consequence (Wacker et al., 1988) as observed in my experiment. Apart from the observed bulging and branching, I see amyloplasts moving to the tip of cells and a disruption of normal plastid positions (Fig 3.7 a, 3.7b, 3.7c, 3.7g & 3.7h). When growth is arrested, it follows that gravitropism is also delayed. Tip growth resumes around 1hr 30 min after oryzalin application and the cell responds to gravity with clear negative gravitropism after 2 hrs 15 min (negative gravitropism in untreated filaments is visible after about 1hr 30 min, (Knight and Cove, 1991). In oryzalin treated cells, amyloplasts aggregate and settle to the bottom flank (Fig 3.7e & 3.7g). MTs appear to be necessary for the maintainance of plastid position and when MTs are depolymerised, plastid sedimentation increases in *Ceratodon* (Schwuchow and Sack., 1993). Since depolymerization of MTs induces plastid aggregation and settling, the MTs would appear to hold the amyloplasts in place in normal *P. patens* and when MTs are disrupted, then settlement occurs. This settling may trigger other receptors to induce signal transduction events. Immunolocalization of oryzalin treated cells revealed a delay in tubulin accumulation. Both 30 min and 60 min gravistimulated filaments had an equal distribution (Fig 3.5 b, c, e, f) of disintegrated MTs. Accumulation of tubulin was clearly

visible (Fig 3.5d) after 4hrs of gravistimulation and a number of MT strands were reformed (Fig 3.5d). This delayed accumulation of MTs may result from delayed gravitropism due to the temporary growth arrest following oryzalin application. Collectively the oryzalin study demonstrates that when MTs are disrupted, changes occur in normal gravitropism and growth patterns of *Physcomitrella* protonema. The growth and MT changes associated with oryzalin treated filaments have been illustrated in a timeline under Fig 4.3.

4.3 Calcium, Gravitropism and Tip growth

Calcium ion modulation has been linked to both gravitropism and tip growth by many observations. A link between intracellular calcium levels and the cytoskeleton has also been indicated by a number of studies. The stability of MTs depends on Ca^{2+} concentration (Raff, 1979). Elevated cytoplasmic calcium levels are known to cause fragmentation of actin filaments and depolymerize MTs (Cyr 1994). MT disruption opens Ca^{2+} channels in *Arabidopsis* /carrot cells and the *Arabidopsis* ton mutant with constitutively disorganized cortical MTs has been shown to have high calcium channel activity (Thion et al., 1996, 1998). Since MTs are potential regulators of calcium channels (Thion et al., 1998) and MT depolymerizing agents lead to loss of directionality and multiple growth points (Wacker et al. 1988; Doonan et al, 1988; Schwuchow et al. 1990; Bibikova et al. 1999), MTs can also control tip growth. A tip focused Ca^{2+} influx is observed in growing filaments of *P. patens* (Allen et al., 2003). This observation had been noticed in other tip growing systems as well (Hepler et al., 2001). Using a Ca^{2+} sensitive vibrating probe, it has also been shown that Ca^{+2} influx occurs in the upper flank of dark grown gravistimulated caulonemal filaments of *P. patens* at around 20 min

(Allen et al., 2003). The timing of this influx coincides with the tubulin accumulation that starts after 30 min. The influx extends about 60 μm along the upper flank and the peak of the influx is about 20 μm from the tip. The calcium ion movements in and out of *P. patens* filaments have been recorded and this flux data profile is seen in Fig. 4.1 (taken from Allen et al, 2003). The maximum influx of calcium does not occur at the tip, but occurs at a distance from the tip of the cell similar to that seen for tubulin accumulation. It is striking that the tubulin accumulation in the lower flank and Ca^{+2} influx in the upper flank of *P. patens* apical filaments correlate in space and time. This correlation has been illustrated in a timeline under Fig 4.2. It can also be said from my growth study data that MTs may control tip growth in *P. patens*, as MT disruption leads to temporary growth arrest and delayed gravitropism.

4.4 Proposed model

Collectively my results and the calcium data (Allen et al., 2003) would indicate that there is a close relationship between MT reorganization and differential opening of calcium channels in *P. patens*. But only if one could image the calcium changes relative to dynamic changes in the microtubule pattern would it be possible to say what is cause and what is effect. Unfortunately, ratio imaging techniques cannot be used to image gravitropic events as the phototropic calcium rise overrides any signal from gravitropic events. (Tucker, unpublished data). So based on available data I propose the following model for gravitropism in *P. patens*.

The gravity signal may generate a tension on microtubules (maybe through amyloplasts). This tension may be sensed by a receptor leading to opening of calcium channels in the upper flank or may stimulate phosphorylation of a calmodulin binding

protein resulting in calmodulin release [higher calmodulin concentrations have been observed in root columella cells, (Allan and Trewavas, 1985)]. Either one of these situations may lead to localized increase in cytosolic calcium concentration. This increased concentration of calcium ion may cause MT disruption in the upper flank. These MTs could relocate to the bottom of the cell and may be visualized as increased fluorescence. Increased MT concentration in the lower flank may lead to an increase in movement of Golgi vesicles containing cell wall materials along these microtubules and to new sites. These vesicles may deposit additional growth materials at the lower side near the tip leading to an increased growth in the lower flank as compared to the upper flank. Fig 4.4 is a schematic representation of the above mentioned interpretations.

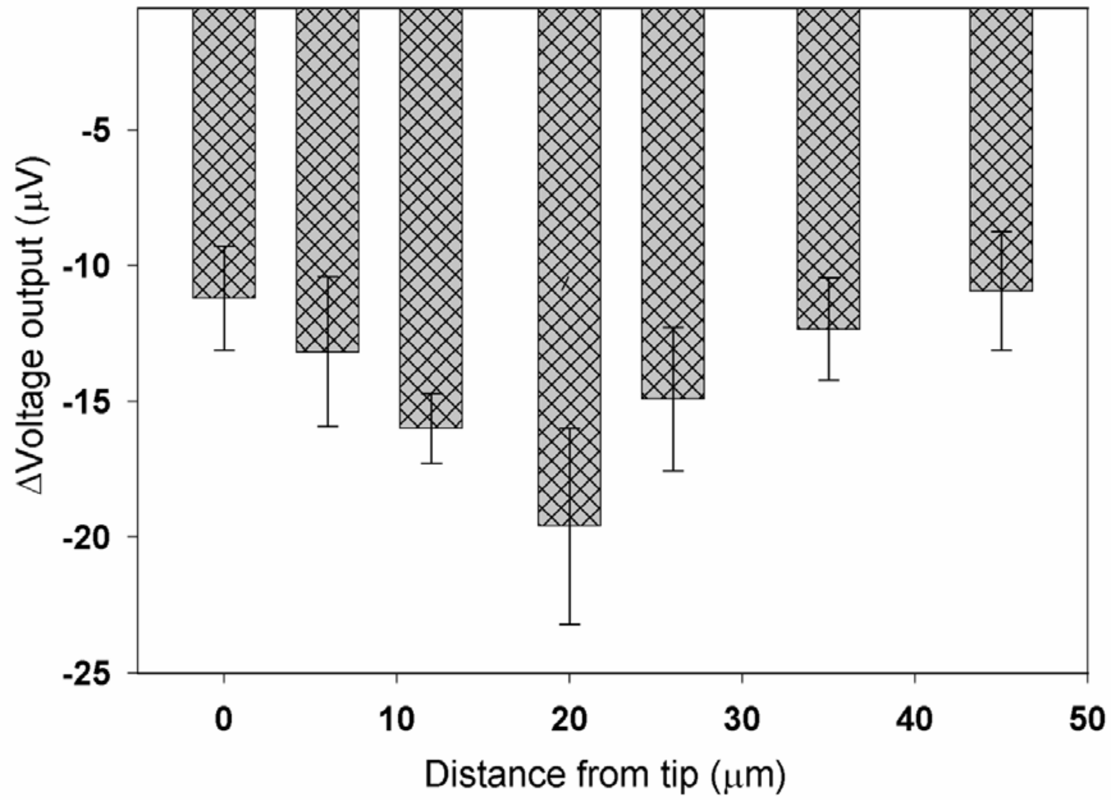

Fig 4.1 Calcium influx in apical caulonemal cell of *P. patens*

Vibrating probe analysis revealed a calcium influx at the tip of a caulonemal apical cell of a gravistimulated *P. patens* filament (taken from Allen et al., 2003).

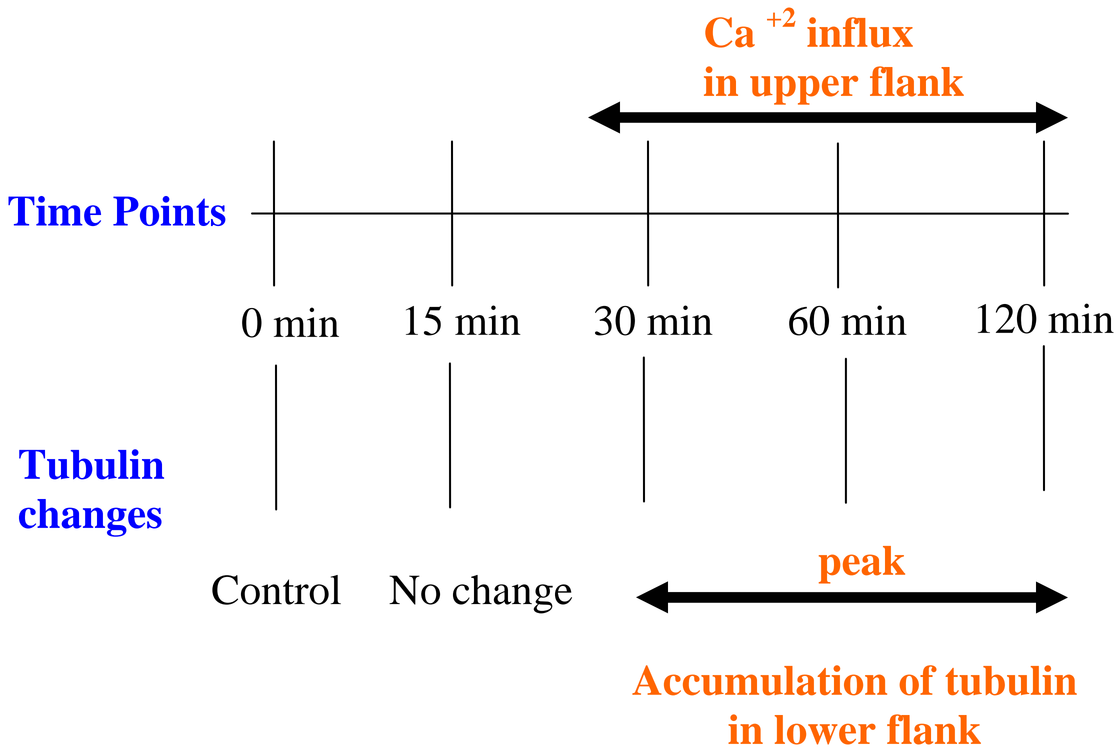

Fig4.2 Summary timeline displaying the changes in tubulin intensity and calcium fluxes over time in gravistimulated filaments

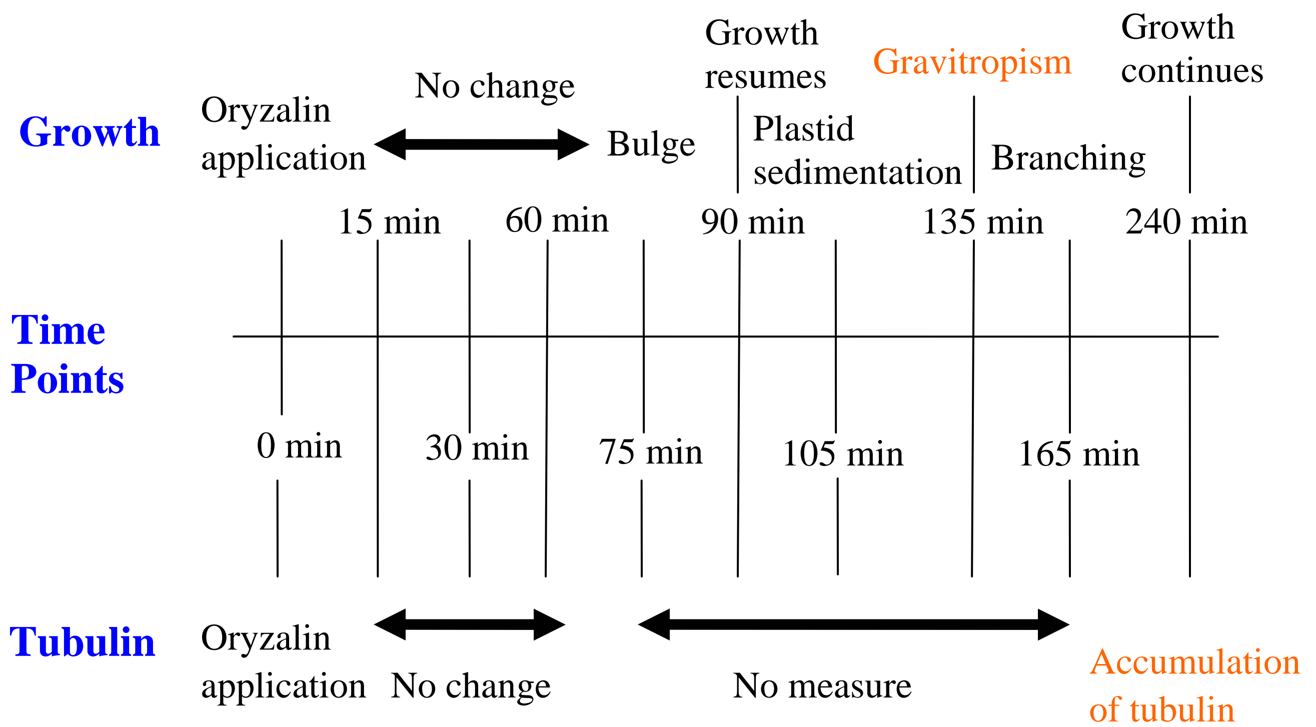

Fig 4.3 Summary timeline of growth and tubulin changes associated with oryzalin treated gravistimulated filaments

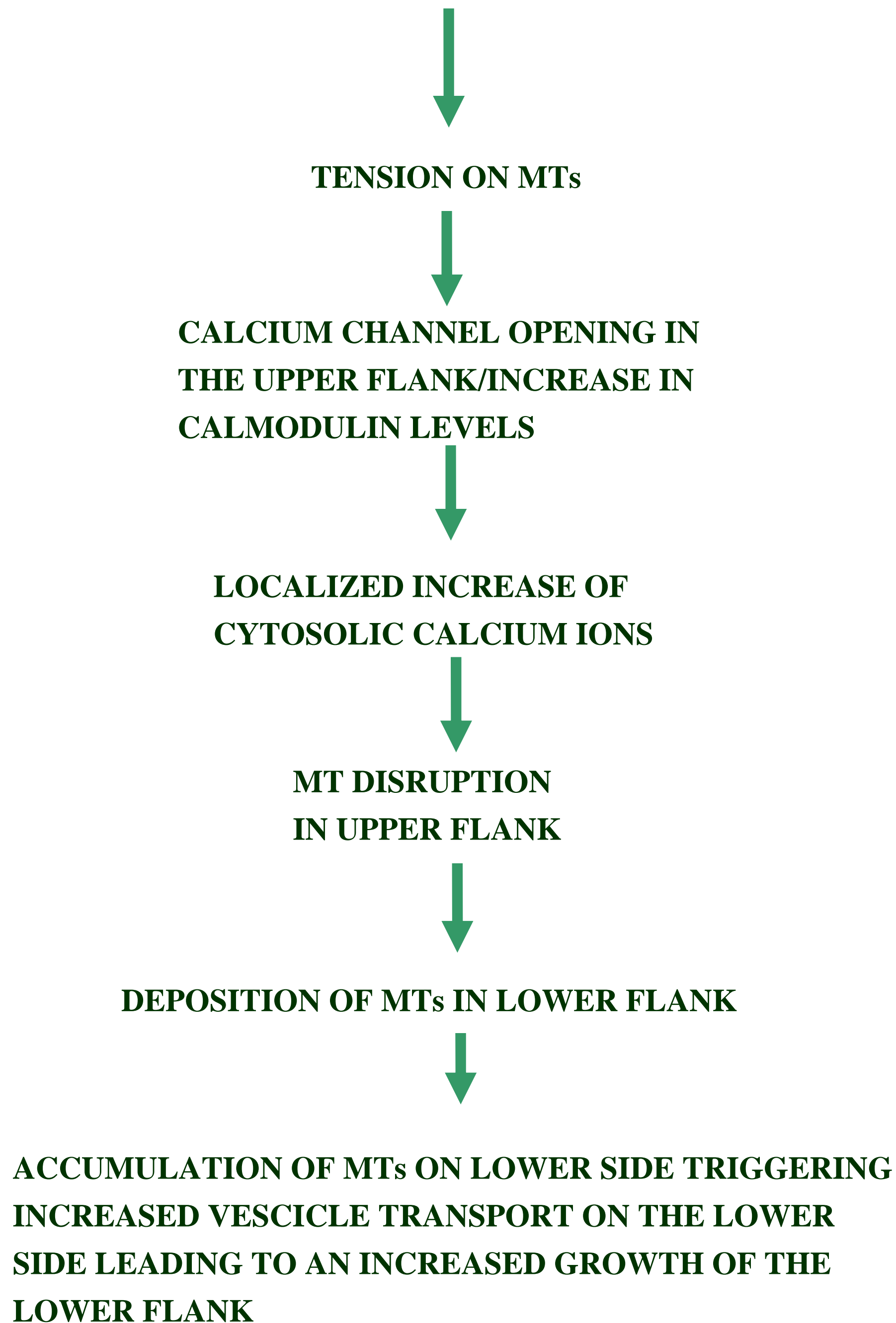

Fig 4.4 Schematic representation of one possible interpretation of gravitropic events in *P. patens* leading to changes in growth patterns.

5 CONCLUSION

It is clear from my experiments that a gravity stimulus is either capable of changing the normal distribution of MTs or alternatively MT changes are essential for signal transduction pathway and disruption of MTs alters tip growth. ***No MT ..No tip growth ..No gravitropic response.***

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