The role of adenylate cyclase-associated protein in higher plant development

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The Role of Adenylate Cyclase-Associated Protein in Higher Plant Development

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Statement of Copyright

Even now
I mind the coming and talking of wise men from towers
Where they had thought away their youth.

E. POWYS MATHERS.

Acknowledgements

This thesis represents four of the most interesting years of my life and as such there are many people whom I wish to thank. Firstly Professor Patrick Hussey for taking me into his laboratory and for providing me with ideas, encouragement and support; my research has benefited immensely form his enthusiasm and knowledge. Secondly I would like to thank all past and present members of the Plant Molecular and Cell Biology Laboratory at Durham for their assistance and companionship. Special thanks must go to Andrei, Ellen, Tijs, Mike and Luisa for their direct help. The help of Sutherland Maciver of the University of Edinburgh and Barry Causier of the University of Leeds was also invaluable to the work presented here.

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Simon Dimmock

Abstract

The Actin Cytoskeleton is essential for Eukaryotic life and is involved in a diverse range of cellular functions. Cyclase Associated Protein (CAP) was first identified in yeast as a regulator of the *CYR1* Adenylate Cyclase. Subsequently CAP family members have been identified in every Eukaryotic kingdom and have also been implicated in the regulation of Actin dynamics. It has been proposed that the CAP family promotes the recycling of Actin monomers by co-operating with members of the Profilin and Actin Depolymerising Factor families.

This study represents an attempt to investigate the function and developmental role of AtCAP1, an *Arabidopsis* member of the CAP family. *Arabidopsis thaliana* is widely used as a model for higher plant development due to its small sequenced genome and the availability of a wide variety of mutants. The elimination of AtCAP1 expression results in a distinct developmental phenotype. Early characteristics include the absence of the root hair collar, reduced root hair initiation and extension. Later onset phenotypes include reduced plant height and a severe reduction in pollen viability. *In vivo* studies of the CAP-deficient cytoskeleton reveal a distinct loss of fine filamentous Actin and the appearance of dense Actin aggregates. Cell expansion is also significantly reduced.

The interaction between AtCAP1 and F-Actin is demonstrated *in vitro* by a biochemical interaction study and a filament bundling activity is suggested. The multimerisation of AtCAP1 and its interaction with other components of the Actin Cytoskeleton are demonstrated via Yeast Two Hybrid interactions.

It is concluded that AtCAP1 is essential for the organisation of the plant cells F-Actin network and that this in turn is required for correct growth and development. It is hypothesised that AtCAP1 function is mediated by regulating the interaction between F-Actin and other Actin-interacting proteins.

LiAc	Lithium Acetate
Kb	Kilo Base
K Da	Kilo Dalton
mRNA	Messenger Ribonucleic acid
N-Terminal	Amino Terminal
PAGE	Polyacrylamide Gel Electrophoresis
PAUP	Phylogenic Analysis Using Parsimony
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline+ Tween 20
PCR	Polymerase Chain Reaction
PEG	Polyethyleneglycol
PFN	Profilin
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
TAE	Tris Acetate +EDTA
TE	Tris +EDTA
Taq	*Thermus aquaticus* Polymerase
TBS	tris Buffered Saline
TBST	Tris Buffered Saline +Tween 20
TDNA	Transfer DNA
TEM	Transmission Electron Microscopy
TEMED	NNN'N'-Tetramethylethlenediamine
Tris	Tris-(hydroxylmethyl)-aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
WT	Wild-Type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

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Chapter I: Introduction

1.1 Overview of the Plant Cytoskeleton

The formation of a highly organised and adapted multi-cellular organism, such as a higher plant, requires a carefully orchestrated program of both cell division and differentiation. This is perquisite for the formation of specific tissues and organs. The cytoskeleton plays a key role in enabling growth and development by influencing the position and plane of cell division, determining cell polarity, permitting cell expansion and enabling differentiation. Regulation of the plane of division is especially important to higher plants as their rigid cell walls prevent cell migration; a common mechanism in animal development.

The cytoskeleton of a higher plant is similar to that of other Eukaryotes in that it is comprised of two major separate and distinct networks, the Actin and Microtubule cytoskeletons. Each comprise of a polymerised monomer (Actin and Tubulin respectively) and an extensive array of associated proteins. The Actin network consists of microfilaments with a diameter of between 7 and 9 nm whereas tubulin polymerises to form hollow tubules with a diameter of 25nm. Both polymers may form bundled fibres, two-dimensional networks and three-dimensional gels with different architectures having distinct physical and functional properties. In addition both networks play a key role in regulating intra-cellular transportation by providing a scaffold for motor proteins (Kinesins, Mysosins and Dyneins) to follow. It is believed that there is a reasonable amount of interaction between the actin and microtubule networks as they co-ordinate to perform various functions including the expansion of tip growing cells, which is discussed in detail within section 1.3.3.

A third network of Intermediate Filaments; so called because their diameter lies between that of Actin and Microtubules, is found within the cells of animals and yeast. Such a system has not been characterised within higher plant models although a protein that cross-reacts with anti-Animal Intermediate filament anti-bodies and assembles into 10nm filaments has been identified (Hargreaves *et al* 1989). There is a paucity of information regarding both the diversity of Intermediate Filaments within the plant kingdom and the role that they may play within the plant cell.

Until recently, it was believed that possession of an organised cytoskeleton was a unique characteristic of Eukaryotes, a view countered by the discovery of both actin and tubulin homologs in a range of prokaryotes (reviewed in Caballido-Lopez and Errington 2003). The actin homolog MreB shares some of the roles played by eukaryotic actin, including the regulation of cell shape and the ability to form filaments in a manner similar to the Eukaryotic protein. (van den Ent *et al* 2001).

1.2.1 An Introduction to Actin

Actin filaments and their associated interacting proteins have been identified in all eukaryotic kingdoms and it has been estimated that they may constitute up to 25% of the total protein content of a mammalian non-muscle cell (Pollard 1993). This suggests that Actin is both ancient, as it must have developed before eukaryotic diversification, and that it plays a fundamentally important role in cellular function.

The incubation of plant protoplasts with a detergent solution is a common method used for the production of complete cytoskeletons and was instrumental in the discovery of higher plant actin. The solubilisation of Carrot (*Daucus carota*) protoplasts and subsequent electron micrography of the cytoskeletal remains revealed the existence of a network of 7nm micro-filaments associated with the nucleus and the substratum (Powell *et al* 1982). Treatment of the protoplasts with microtubule depolymerising drugs, such as Colchicine, did not affect the presence of these filaments. The size of the filaments and their association with the nucleus resulted in them being classified as representing a higher plant actin cytoskeleton.

The actin filament is formed from a chain of 42kDa actin monomers, which are globular proteins and such are referred to as G-actin. The polymerisation of actin filaments will be discussed fully in section 1.2.3 but in essence an actin filament is a helical string of G-actin monomers with a diameter ranging from 5-9 nm. Each monomer is both rotated by 166° and translated by 2.75nm in relation to the previous monomer; meaning that F-actin appears to have a double-stranded structure. A diagrammatic view of an actin filament is given in figure 1.1. The incubation of actin filaments with the S1 head

Figure 1.1: The Arrangement of an Actin Filament

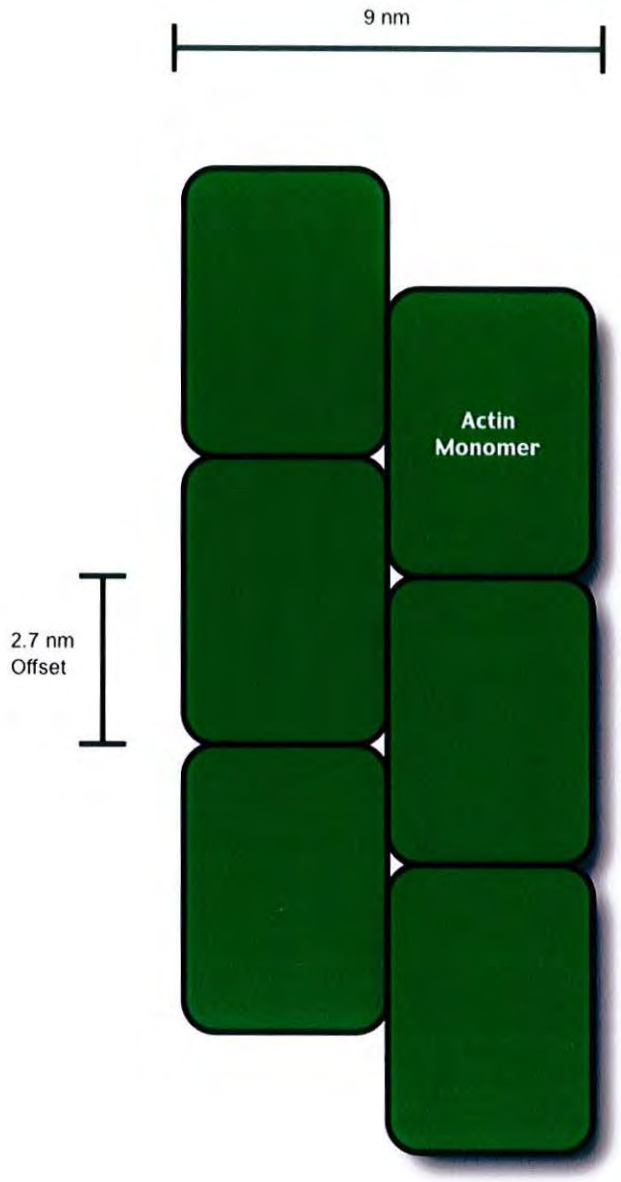


Fig 1.1 The Filament is arranged as a left-handed double-helix

of myosin results in the formation of 'arrow-heads' upon the filament, with all of the heads being oriented in the same direction. This demonstrates that actin filaments posses polarity and has led to the ends of the filament being described as being barbed or pointed.

A monomeric molecule of G-actin consists of a single polypeptide chain, a nucleotide (either ATP or ADP) and an associated divalent cation. The tendency of actin to polymerise initially made it impossible to grow crystals of pure globular actin, which resulted in the monomer's structure remaining unsolved. It was however found that G-actin was able to form a 1:1 complex with Bovine Pancreatic Deoxyribonuclease I (DNAse I) and that this form was more amenable to crystallisation (Kabsch *et al* 1990). The protein was found to consist of two domains of roughly equal size, each of which split into two sub-domains. Both the associated adenine nucleotide and the divalent cation (either Mg^{2+} or Ca^{2+}) were found to be held within a cleft between the two domains. The high affinity cation-binding site was localised to a deep hydrophilic pocket between the adenine nucleotides phosphate groups and three G-actin residues (Asp11, Gln 137 and Asp 154) (Kabsch *et al* 1990). Later the actin binding drug Latrunculin was used to inhibit polymerisation (Morton *et al* 2000). The structure of this 1:1 Actin:Latrunculin complex was in turn solved (Yarmola *et al* 2000).

1.2.2 The Formation of Actin

The formation of actin filaments is a complicated multi-phasic process consisting of two major steps, the energetically unfavourable initiation of new filaments (Nucleation) and the energetically favoured addition of monomers to the filament ends. A generalised time course for the formation of an actin filament is shown in Figure 1.3.

1.2.2.1 Actin Nucleation

The *de novo* initiation of actin filaments first requires the creation of a nucleus of at least three monomers, this is energetically unfavourable and so requires the involvement of an actin-nucleating protein (Dos Remedios *et al* 2003 and Winder and Ayscough 2005). There are three distinct families of proteins with a proven ability to nucleate actin filaments; the Arp2/3 complex,

Fig 1.2: The Dynamics of Actin Filaments

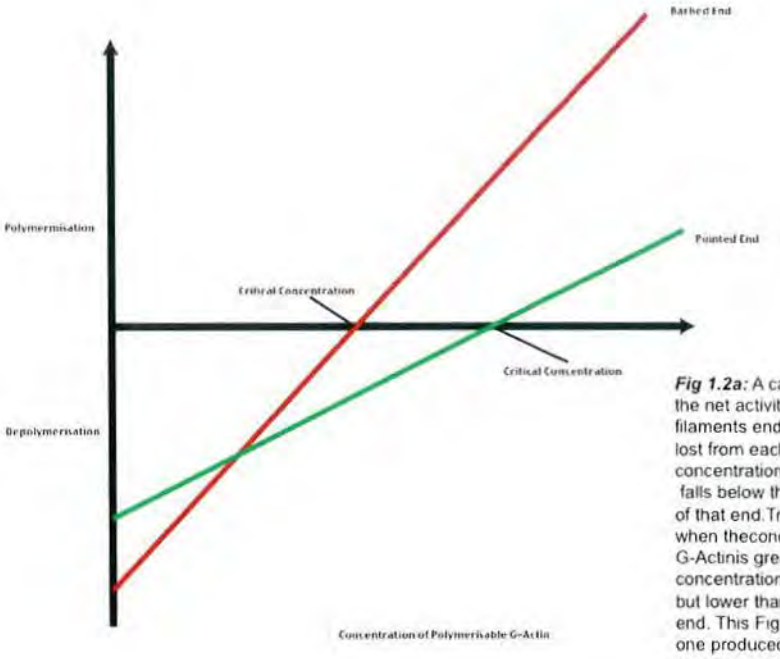


Fig 1.2a: A cartoon graph showing the net activity at each of the filaments ends. Monomers will be lost from each end when the concentration ofavailable monomer falls below thecritical concentration of that end.Treadmilling will occur when theconcentration of available G-Actinis greater that the critical concentration of the barbed end but lower than that of the pointed end. This Figure is derived from one produced by Dr.Sutherland Maciver and is used with permission.

Fig 1.2b: Treadmilling is a product of the constant addition of monomers to the barbed end and their subsequent disassociation from the pointed end. An individual monomer will, in effect, pass down the filament before the hydrolysis of its bound ATP molecule provides the energy to permit its disassociation.

Figure 1.3: The Formation of an Actin Filament

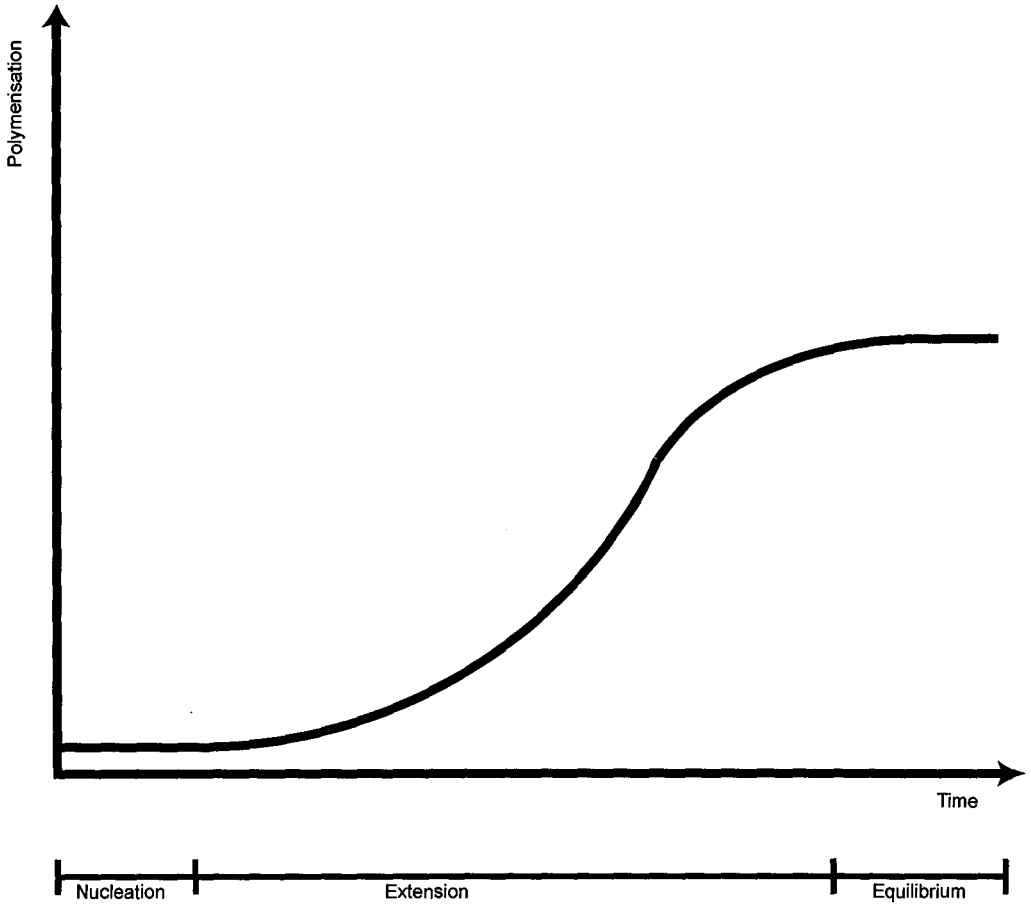


Fig 1.3: Polymerisation is preceeded by an energetically unfavourable nucleation stage during which actin trimer formation will be induced by a number of nucleating proteins. This represents a lag phase. This is followed by the net addition of monomers to the filament and therefore its extension. Addtional monomers are added singularly in an energetically favoured reaction. Eventually a new equilibrium will be reached where monomer addition and loss will be balanced. This is referred to as treadmilling and the result is no net change in filament length

the Formin family and the recently discovered *Drosphila* protein Spire. There are differences in the architecture of the filaments produced by the three pathways but all three mechanisms are dependant upon a sufficient number of actin monomers being brought into close proximity.

The Arp2/3 complex is a group of seven proteins (Machesky and Pollard 1992), which as a cluster, may interact with an existing actin filament and initiate the formation of a new filament at angle of 70° from the initial filament. This results in the formation of the complex dendritic networks of actin that are found within filopodia and lamellipodia and are responsible for the formation of actin 'comet-tails', which are used by the pathogenic bacteria *Listeria* (Tilney and Portnoy 1989) and *Shigella* (Goldberg and Sansonetti 1993) to migrate through host cells. Two members of the complex, i.e. Arp's 2 and 3, show a close homology to the actin monomer (Machesky *et al* 1994) and it is believed that these two subunits mimic an actin dimer, to which a single monomer has to be added to form the trimer nucleus (Volkmann *et al* 2001). It is hypothesised that the complex then caps the pointed end allowing the addition of monomers to the barbed end and rapid elongation.

Arp2/3 is regulated by a diverse array of interacting proteins; its activity is stimulated by interaction with members of the Wiskott-Aldrich Syndrome Protein (WASP) family and with Hemopoetic Stem/Progenitor Cell Clone 300 (HSPC300). Its activity was believed to be inhibited by interaction with three proteins PIR131, NAP125 and ABI2 but there is conflicting evidence that the three proteins may instead also directly stimulate Arp2/3 to nucleate new filaments (Innocenti *et al* 2004). Only some members of the Arp2/3 pathway have been identified within higher plant models (reviewed by Deeks and Hussey 2003) but there is some evidence that they function in a manner similar to their animal and unicellular homologs. Firstly the disruption of the HSPC300 homolog BRICK1 in *Zea mays* results in a loss of diffuse actin networks at the growing lobes of epidermal cells (Frank and Smith 2002) and secondly the knock-out of NAP1 in *Arabidopsis* also resulted in alterations to actin architecture and general cellular morphology of trichomes (Deeks *et al* 2004).

The Formin family are believed to be responsible for the generation of the long linear actin filaments that comprise actin cables (reviewed by Winder and Ayscough 2005). The animal formins include three conserved Formin-Homology (FH) domains that are believed to be essential for the protein's

function. Mutagenesis and deletion studies have shown that the FH1 domain is required for interaction with the actin-monomer binding protein Profilin (discussed in 1.2.5.2), the FH2 domain is critically important for the nucleation of new filaments and the FH3 domain is required for the proteins correct localisation (reviewed by Deeks *et al* 2002). The complete genome of *Arabidopsis* contains 17 genes that contain sequences similar to both the FH1 and FH2 domains although there are no close matches to the FH3 domain. These genes can be divided into two large groups, the Type I formins that include a putative trans-membrane domain and are believed to be localised to the membrane and the Type II formins, for which localisation in unclear. The large multigenic nature of Formin families has made study of their *in vivo* role troublesome as there is believed to be a high degree of redundancy between family members.

The third major mechanism of actin nucleation is catalysed by the *Drosphila* protein Spire which contains four separate actin monomer binding WH2 domains but is yet to been identified within the genome of a higher plant . It has been shown that the Spire rapidly induces the polymerisation of actin filaments independently of Arp2/3 but does not accelerate the elongation of pre-formed seeds (Quinlan *et al* 2005); this is symptomatic of *de novo* filament formation. The exact mechanism of Spire action is currently unknown but it is believed that the binding of monomeric actin to two of the WH2 domains results in these monomers being brought together resulting in the formation of a dimer. The other two WH2 domains will then be responsible for the addition of a further two monomers (Quinlan *et al* 2005). The organisation of the filaments generated by Spire has not been extensively investigated but the examination of the nuclei with electron microscopy has revealed them to be short linear rods. Spire does not co-sediment with F-actin making it unlikely that it induces branching from existing filaments.

1.2.2.2 Factors Affecting the Polymerisation of Actin.

The growth of actin micro-fibrils is mediated by the addition of monomers to an existing filament (or to a newly nucleated seed). The rate of elongation

was found to be directly proportional to the concentration of available monomer leading to the belief that monomers are added singularly (Oosawa and Asakura 1975). G-actin molecules may be added to either end of the filament but it was found that there was a significantly higher rate of addition to at the barbed end than at the pointed end (4 to 8 times) with actin derived from muscle tissue (Woodrum *et al* 1975). A similar disparity between the filament ends was seen when non-muscle actin was used (Tilney *et al* 1981) and there is little reason to believe that plant actin would have dissimilar characteristics.

Biochemical measurements and quantitative analysis of electron micrographs have revealed the actin filament to be a highly dynamic structure with association and dissociation of monomers from both ends of the filament. The observation that monomers would only be added to both ends of the filament, as opposed to just the barbed end, when a significantly higher concentration of G-Actin was available (Woodrum *et al* 1975) has led to the belief that the two ends have differing critical concentrations. That is to say that the concentration of available G-actin at which the rate of association and disassociation from the polymer is equal (the equilibrium point) is lower for the barbed end than it is for the pointed end resulting in a lower threshold for polymerisation at the barbed end. This is illustrated in Figure 1.2.

Further experiments have revealed that several factors influence the rate of polymerisation at either end of the filament, including the identity of both the nucleotide and cation associated with the monomer. Analysis of addition to both the barbed and pointed ends revealed that ATP-Actin was incorporated into the filament at a significantly higher rate than was ADP-bound actin (Pollard 1986). Again a far greater rate of polymerisation was observed at the barbed end. Interestingly this study revealed that both ATP and ADP-bound actin also disassociated from the barbed end at a greater rate than for the pointed end, although the difference between the ends was approximately 8-fold greater for ADP-Actin.

All of the aforementioned observations had been made using Mg^{2+}-associated actin, but it is known that actin may also bind Ca^{2+} ions within its inter-domain cleft and that its affinity for Ca^{2+} ions is several fold greater than for Mg^{2+} (Gershman *et al* 1986). Polymerisation studies with Ca^{2+}-Actin have revealed an increase in the critical concentration at the barbed end (therefore a higher concentration of monomer is required for net elongation from this end)

and a slight decrease in the critical concentration at the pointed end (Bonder *et al* 1983).

The physiological relevance of these observations is not entirely clear as the intra-cellular concentration of Ca^{2+} within the plant well is a thousand-fold less than that of Mg^{2+} (1μM as opposed to 1mM) (Vantard and Blanchoin 2002), so it is likely that the Mg^{2+} bound form is more abundant *in vivo*. It is, however, known that Eukaryotic cells sequester Ca^{2+} within various organelles (especially the Endoplasmic Reticulum) and that this may be released in response to signalling events leading to a high local Ca^{2+} concentration. A change in actin polymerisation kinetics is a possible outcome of such signalling events. An alternate possibility is that the uptake of Ca^{2+} ions by monomers affects the interaction between G-actin and actin binding proteins.

1.2.2.3 A generalised model of an Actin Filament.

Consideration of the factors described in the previous section allows us to form a generalised model for the polymerisation dynamics of an actin-filament. Within a cell there will be an equilibrium between filamentous and globular actin; net polymerisation would be induced if the equilibrium shifted towards filamentous actin (i.e. if there was an increase in the concentration of available polymeriseable actin). Addition of monomers to preformed filaments is rapid, as polymerisation is energetically favourable and filament elongation will proceed until a new equilibrium is achieved (i.e. until the supply of polymerisable actin monomers has fallen below the critical concentration of the barbed end).

Hydrolysis of each monomer bound ATP molecule follows polymerisation although this step is not essential for addition as ADP-G-Actin can also be incorporated into a filament, albeit at a slower rate (Pollard 1986). The loss of the bound ATP molecule is believed to occur in a bi-phasic process with the initial hydrolysis being followed by the release of an inorganic phosphate molecule (Pi). The energy produced by the loss of the Pi destabilises the filament and encourages monomer release (Korn *et al* 1987). This increases the rate of monomer turnover and so promotes dynamism within the system.

The addition and removal of monomers from the ends will result in an individual monomer progressing along the filament towards the pointed end in a

process known as 'Treadmilling' (Wegner 1976). This will only occur when the concentration of available monomer is in excess of the barbed end's critical concentration but is less than the critical concentration of the pointed end. The hydrolysis of the monomers associated nucleotide will occur during its progression along the filament with the result being the formation of a subunit with both a bound ADP and inorganic phosphate (Pi) molecule (Carlier 1991). Eventually the Pi will disassociate leaving an ADP-bound monomer within the filament . A diagrammatic representation of this process is shown in Fig 1.3.

1.2.3 Plant Actins

There are ten actin genes within the *Arabidopsis* genome, of which eight have been shown to be expressed, with the other two believed to be pseudo-genes (McDowell *et al* 1996). There is far greater diversity amongst the *Arabidopsis* actins than there is amongst vertebrate actins, as the *Arabidopsis* family displays multiple non-conservative substitutions, whereas the six vertebrate actins (including both muscle and non-muscle isoforms) have none (Meagher *et al* 1999). Moreover the pI's of the vertebrate actins are confined within a 0.3 pH unit range whereas the *Arabidopsis* actins vary over 0.7 pH units (McLean *et al* 1990). This is indicative of significant biochemical differences between the isoforms as even the relatively minor conservative substitutions of vertebrate actins lead to distinct differences in polymerisation (Rubenstien 1990) and protein-protein interactions (Oshima *et al* 1989)

Expression of the different isoforms seems to be both tissue specific and show some dependence upon the developmental stage of the plant (Meagher *et al* 1999). *ACT2* and *ACT8* are expressed in most vegetative tissues and are joined by ACT7 in younger vegetative tissue. *ACT1* and *ACT3* are expressed in young ovules and pollen grains and so are thought to represent a class of reproductive actins. In addition *ACT4* and *ACT12* are expressed at specific times throughout pollen development and *ACT11* has also be shown to be expressed in pollen. Interestingly all eight genes are expressed in developing vascular tissue and some of the reproductive actins also seem to play a role in organ primordia (Meagher *et al* 2000).

The expression patterns would suggest that many cell types will contain multiple actin isoforms, which leads to the possibility of isovariant dynamics and

therefore increased flexibility. An example of this would be the formation of heteropolymers, which may show differing kinetics, bundling properties and response to regulatory inputs from a filament comprised solely of one actin isoform. Other possibilities include differing expression of actin isoforms throughout the cell cycle or differential localisation of isoforms within the cell (so as to permit the formation of different actin architectures).

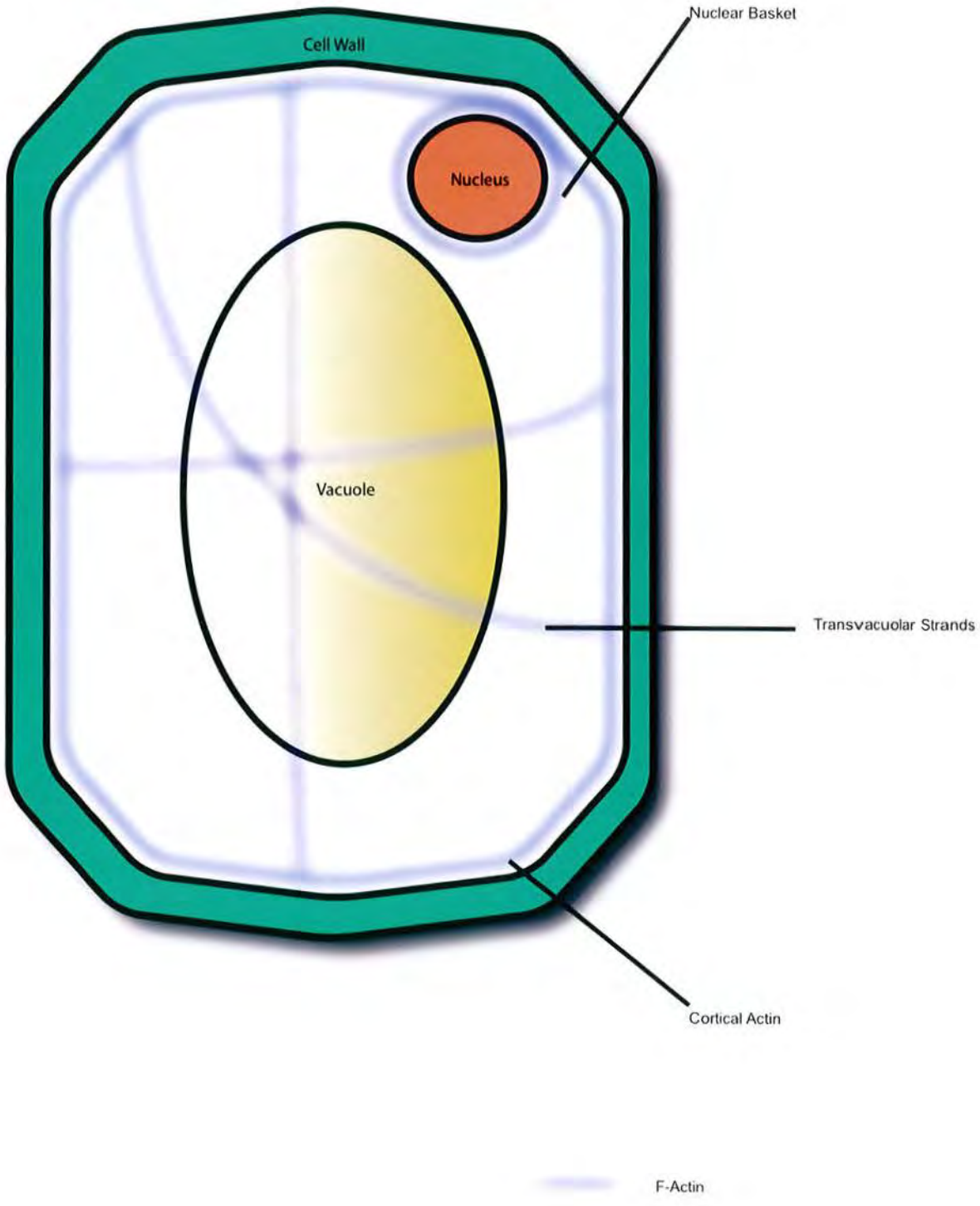
1.2.4 The Localisation of Actin Within the Cell.

An understanding of the role played by complex actin structures first requires that the *in vivo* location of such networks is identified and that any changes throughout cell division, growth and development are studied. The incubation of Alfalfa (*Medicago sativa*) suspension cells with Rhodamine-conjugated phalloidin allowed the distribution of actin filaments within the interphase cell to be studied (Seagull *et al* 1987). Three major networks were identified: a fine network of cortical actin adjacent to the plasma-membrane, a basket of filaments surrounding the nucleus and collection of large bundles adjacent to the vacuole. The 'nuclear-basket' was found to associate with the nuclear membrane and to extend into cytoplasmic strands, these are thought to provide spatial guidance for cytoplasmic streaming (reviewed by Shimmen and Yokota 2004)

Progression through mitotic division results in a reduction of the intensity of actin staining and a loss of these distinct structures. The cortical actin network and vacuolar bundles are not observed after early prophase and eventually the nuclear basket is reduced to a non-filamentous diffuse glow when stained with rhodamine-conjugated Phalloidin. Filamentous actin has been identified within the nuclear spindle (in close association with Microtubules) during metaphase and anaphase (Forer and Jackson 1979) although the cytoplasm still lacks identifiable filamentous actin at this stage (Seagull *et al* 1987).

The onset of Telophase is marked by the appearance of the phragmoplast, a microtubule-based structure that demarks the location of the new cell wall. The phragmoplast was found to stain brightly for actin filaments and it has been shown that the Cytochalasin-D mediated disruption of Actin filaments results in abnormal phragmoplast formation (Gunning and Wick

Figure 1.4: Actin Within the Plant Cell



1985). The nuclear-basket is also seen to gradually reform around each of the daughter nuclei at this time point prior to the transvacuolar strands reforming in late telophase. The reformation of the cortical actin array can take much longer and distinct cortical filaments are often not discernable until early G1 phase (Seagull *et al* 1987).

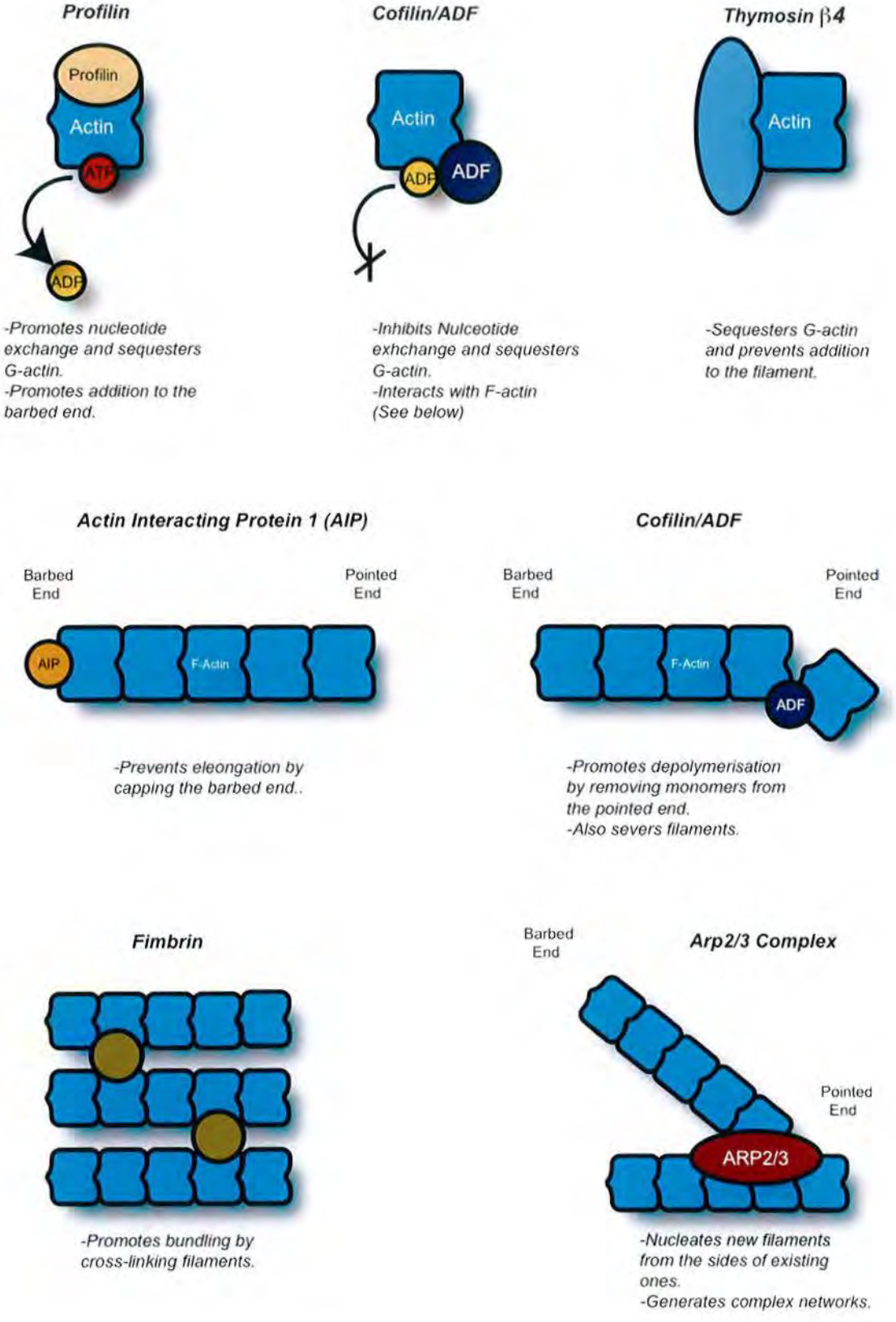
1.2.5 An Overview of Actin Binding Proteins

The maintenance and regulation of a dynamic and effective actin cytoskeleton requires a diverse group of actin-associated proteins. It has been estimated there are at least one hundred and sixty two distinct actin-associated proteins throughout the *Eukaryote* (Dos Remedios *et al* 2003). This figure would have to be increased significantly if isoforms were to be included as many families contain multiple distinct members. A diverse range of activities are demonstrated by the actin interacting proteins including filament cross-linking (e.g. Spectrin), monomer sequestering (e.g. Thymosin), Filament Capping (e.g. Gelsolin), filament severing (e.g. Villin) and de-polymerisation promoting (e.g. Cofillin). Additional actin-associated proteins include the aforementioned nucleating proteins (i.e. Arp2/3, Formin and Spire) and the motor proteins that drive cytoplasmic streaming (e.g. Myosins).

The importance of actin-associated proteins can not be overstated. It is interesting to note that the typical intracellular concentration of actin monomers is significantly greater than the critical concentration of both the pointed and barbed ends. Therefore the activity of G-actin sequestering proteins is essential in preventing all of the available monomer from becoming spontaneously incorporated into filaments. In doing so they help to generate a cytoskeleton that is dynamic and therefore responsive. It has been estimated that the *in vivo* turnover of actin monomers is some 100 to 200 fold greater than that observed *in vitro* in the absence of Actin-interacting proteins (Theriot 1997). This simple observation demonstrates the importance of the role played by actin modulating proteins, such as ADF (to be discussed in section 1.2.5.1) in promoting dynamism within the actin system.

A description of the actin-associated proteins relevant to the activity of the *Arabidopsis* cyclase-associated protein (CAP) and the work presented in

Figure 1.5 The Activities of Selected Actin Assosciated Proteins



this thesis will be given in the next few sections, but it is beyond the scope of this chapter to give a full description of all higher-plant actin-associated proteins. Interested readers are directed towards the reviews of Dos Remedios *et al* (2003), Winder and Ayscough (2005) and Pollard and Borisy (2003). Information specific to the plant actin cytoskeleton is given in Staiger *et al* (2000).

1.2.5.1 Actin Depolymerising Factor/Cofilin

Actin Depolymerising Factor (ADF) is small actin-binding protein (typically 15-22KDa) that is found across the Eukaryotic kingdoms, with most species expressing multiple isoforms (reviewed in Kovar and Staiger 2000). ADF was originally isolated from a chick-brain extract and was found to form a 1:1 complex with G-actin (Bamberg 1980). Cofilin was extracted from Porcine brain and was found to co-sediment with F-actin (Nishida *et al* 1984). Sequencing of both proteins revealed a high degree of similarity leading to them being grouped into the ADF/Cofilin family of actin-associated proteins. A typical characteristic of this family is the ability to interact with both G and F-actin and a tendency to promote the rapid depolymerisation of monomers from the pointed end (reviewed by Kovar and Staiger 2000). There is diversity in biochemical properties amongst the family (the co-sedimentation of some members with F-actin is explained by a low catalytic activity) and diversity in response to regulatory mechanisms.

The importance of ADF to cellular function is demonstrated by the lethal effects of its knockout upon both *Drosophila* (Gunsalus *et al* 1995) and the fission yeast *S.pombe* (Moon et al 1993). It has been measured as having a concentration as great as 20μM in some vertebrate cells, which was estimated to represent some 2% of the total soluble protein and one third of the total amount of actin present (Koffer and Daridan 1985), which is suggestive of significant importance to cellular function. Initial evidence of its importance to *in vivo* actin dynamics came from its localisation to dynamic actin rich regions of the cell, including the leading edge of motile cells (Bamburg and Bray 1987). Its relevance to the plant actin cytoskeleton is demonstrated by the rapid cessation of cytoplasmic streaming F-actin re-arrangement provoked by its micro-injection into *Tradescantia* cells (Hussey *et al* 1998).

Members of the ADF/Cofilin family all possess a fold that contains two specific actin-binding sites. Mutagenesis studies have suggested that one of these is required for both G and F-actin binding, whilst the second is involved solely in association with filaments (Jiang *et al* 1997).

The mechanism of de-polymerisation is still not entirely understood but it has been noted that the binding of ADF to F-actin induces a 5° twist between subunits (McGough *et al* 1997). This rotary movement may be enough to significantly weaken the bonds between monomers. The specificity of ADFs activity to the pointed end is explained by it having a significantly higher affinity (80-fold in the case of AtADF1) for ADP-bound actin than it does for ATP-actin (Carlier *et al* 1997). A similar result was obtained when ZmADF3 (from *Zea mays*) was examined (Gungabisson *et al* 1998), so it may be assumed that higher plant ADF's are more likely to interact with subunits proximal to the pointed end (where ADP-actin is most abundant).

Nucleotide exchange by the released ADP-Monomer is inhibited by the presence of the bound ADF molecule (Nishida 1985) but the equilibrium state would suggest that the two proteins would rapidly disassociate allowing exchange to occur. This is supported by the discovery that the concentrations of both ADF-bound ADP G-actin and ATP-G-actin increase following the addition of AtADF1 (Didry et al 1998). So it can be said that ADF plays an important role in regenerating polymerisable actin monomers and in doing so promotes polymerisation at the barbed end of the filament.

Functional interactions between ADF family members and other actin binding proteins are complex but allow fine regulation of polymerisation and de-polymerisation. For example it is proposed that the capping of barbed ends by Gelsolin will result in the total de-polymerisation of capped actin filaments in an ADF-mediated process. However such a decrease in F-actin would result in considerably more monomer being available for the rapid polymerisation of un-capped filaments, i.e. the products of *de novo* nucleation (Kovar and Staiger 2000), so there may in fact be an increase in localised polymerisation. This model is supported by the observation that ADF (in the presence of Gelsolin) increases the rate of Arp2/3 mediated nucleation (Ressad *et al* 1999) and that ADF, a barbed end capping protein and Arp2/3 were all found to be essential for *Listeria* locomotion (Loisel *et al* 1999). Further synergistic interactions will be mentioned in relation to Profilin (1.2.5.2), AIP, (1.2.5.3) and CAP (1.5.3).

It is also believed that some ADF family members possess a mild filament severing activity (Maciver 1991). This would accelerate actin turnover both by providing more pointed ends for monomers to be removed from and by creating more barbed ends for them to be added to.

In general most ADFs show an increase in de-polymerising activity as the pH increases within physiological limits (Kovar and Staiger 2000), an example being ZmADF2 (Gungabisson *et al* 1998). There are however several exceptions, an example being Actophorin (from *Acanthamoeba*), which shows no pH dependence (Maciver *et al* 1998), but in general control of the intracellular pH will have a regulatory effect upon ADF activity. Protein kinase pathways also play an important role in the regulation of ADF as the phosphorylation of Human Cofilin's Ser 3 residue by a Lim family kinase results in an inhibition of activity by preventing the interaction with actin (Arber *et al* 1998).

There are no close homologs of the Lim kinases in the genomes of higher plants but it has been shown that the phosphorylation of *Zea mays* ADF3 upon Ser 6 (the equivalent of mammalian Ser 3) by a Calcium Dependent Protein Kinase (CDPK) results in a loss of de-polymerising activity (Smertenko *et al* 1998). The same study showed that the replacement of Ser 6 with an Aspartate residue (so mimicking constitutive phosphorylation) also significantly reduced depolymerising activity. So it has been proposed that the gradients of Ca^{2+} found across many plant cells, such as growing root hairs, regulates ADF activity and in doing so spatially regulate actin dynamism within the cell (Hussey *et al* 2004). The binding of additional proteins (such as members of the 14-3-3 family) to phospho-proteins is another common method of phosphorylation dependent regulation (See Roberts *et al* 2003). Indeed it has been shown that a mammalian cofilin interacts with a 14-3-3 protein via its phosphorylated Ser 3 residue (Birkenfield *et al* 2003) and that this prevents dephosphoryaltion and therefore actin depolymerising activity (Gohla and Bokoch 2002).

Phospholipid signalling pathways provide another regulatory input into the ADF/cofilin family as it has been shown that the binding of both PIP and PIP_2 to a site adjacent to the actin binding sites inhibits the mammalian ADF/Actin interaction (Yonezawa *et al* 1990). The work of Gungabisson *et al* (1998) showed that higher plant ADFs (in this case ZmADF2) showed a similar response and so it is believed that the phospholipid response is conserved.

Finally it is important to note that there is often great diversity in both the biochemical activity of a given species various ADFs and in their responsiveness to regulatory mechanisms. Temporal and tissue-specific control of ADF isoform expression is likely to provide an additional global mechanism of actin regulation.

1.2.5.2 Profilin

The Profilins are another family of small (typically 16Kda) actin binding proteins that are ubiquitous throughout the *Eukaryote* (reviewed by Hussey *et al* 2004 and Kovar and Staiger 2000). The first profiln was identified as co-purifying with actin from spleen tissue and was found to bind actin monomers with a 1:1 stoichiometry (Carlsson *et al* 1977). This led to the suggestion that it acted as a G-actin sequestering molecule. The over-expression of profilin in mammalian cells however leads to a net increase in F-actin levels (Finkel *et al* 1994), which is contrary to what would be expected from a monomer sequestering protein. Clearly the function of profilin is more complex than first appeared

The increase in actin polymerisation that corresponded to Profilin over-expression can be explained by two separate activities. Firstly profiln promotes nucletotide exchange by the bound actin monomer and in doing so increases the pool of ATP-G-Actin available for polymerisation (Goldschmidt-Clermont *et al* 1992). Secondly profilin can act as a 'shuttle' molecule that mediates the transfer of actin monomers from Thymosinβ4 (a G-actin binding protein which is thought to inhibit polymerisation) to the growing filament at the barbed end (Pantaloni and Carlier 1993). There is an observed synergistic increase in actin polymerisation when both Profilin and ADF are present as, in effect, both proteins complement each others role and in doing so increase the pool of available polymerisable monomer (Didry *et al* 1998). Profilin does however prevent the addition of monomers to the pointed end of the filament even if the availability of monomers is in excess of its critical concentration.(Kovar *et al* 2000).

The first higher plant Profilin was isolated from Birch pollen and was shown to be able to bind monomeric actin (Valenta *et al* 1993). The sequence

homology between the higher plant Profilins and those of other organisms is low (approximately 25%) but there is functional conservation as their expression in mutant *Dictyostelium* cells lacking profilin resulted in the phenotype becoming complemented (Krakesioglou *et al* 1996). There must however be some significant functional differences as higher plants are believed to lack a close homolog of Thymosinβ4 (Hussey *et al* 2002) and so a barbed end monomer shuttle would be superfluous. In addition studies of profilins isolated from *Arabidopsis thaliana* and *Zea Mays* (Perelroizen *et al* 1996 and Kovar *et al* 2000) have suggested that, at least in vitro, higher plant profilins are unable to promote nucleotide exchange by the bound actin monomer.

These two significant differences have led to the opinion that plant profilns act solely as G-actin sequestering proteins. This view is supported by profilin being found to have a cytoplasmic distribution in Lilly pollen tubes (Vidali and Hepler 1997) as opposed to localising to regions of high actin dynamism. Such localisation would be expected if profilin was promoting polymerisation. Further evidence for a purely sequestering role is provided by the observation that the micro-injection of a Maize Profilin into *Tradescantia* stamen hair cells results in a reduction in the amount of visible F-actin and the termination of cytoplasmic streaming (Staiger *et al* 1994). This is a direct contrast to the aforementioned effect of over-expressed mammalian profilins (Finkel *et al* 1994).

Most plants possess multiple Profilin isotypes with there being variation in their expression program. For example the genome of *Arabidopsis thaliana* contains five distinct Profilin genes, with *PRO1, PRO2 and PRO5* being constitutively expressed and *PRO3* and *PRO4* being confined to pollen (reviewed by Hussey *et al* 2004). It is thought likely that there is variation in activity and regulation of the isotypes so, as with ADF, control of when and where to express specific Profilins permits some global regulation of Profilin activity. The genomes of most higher plants analysed to date contain between five and ten Profilin isoforms (Gibbon and Staiger 2000).

Phospholipid-derived secondary messengers are likely to be involved in the in vivo regulation of Profilin activity as mammalian isoforms have been shown to bind PtdIns(4,5)P_2 (Lassing and Lindberg 1985). This association was found to prevent the hydrolysis of the phospholipid by Phospholipase C (PLC) and would result in the retention of Profilin by the plasma membrane. However

the phosphorylation of PLC by a tyrosine kinase overcomes this inhibition; resulting in the phospholipids hydrolysis and the release of Profilin into the cytosol (Goldschmidt-Clermont *et al* 1990). Such a mechanism seems to be conserved with the higher plant system as a Birch Profilin has also been shown to bind PtdIns(4,5)P_2 and to prevent its hydrolysis by PLC (Drobak and Watkins 1994). This interaction allows for the coupling of Profilin activity (and therefore actin dynamism) to an important intracellular signalling system.

Profilins are known to form complexes with other actin modulating proteins; Arp2/3 for example was initially isolated via its interaction with Profilin (Machesky *et al* 1994). It is reasonable to speculate that in this case Profilin serves to increase the barbed end on-rate of newly nucleated (or branched) filaments. Plans Profilins have also been shown to be able to bind to proteins with poly-proline domains (Domke *et al* 1997). Such proteins include CAP and members of the Formin family although the relevance of these interactions is unclear as Profilin is unlikely to contribute towards promoting polymerisation.

1.2.5.3 Actin Interacting Protein 1(AIP1)

AIP1 was initially identified as a yeast protein with the ability to bind actin via a Yeast-two-hybrid screen (Amberg *et al* 1995) and the same technique has also been used to show that AIP1 interacts with ADF/Cofilin family members (Rodal *et al* 1999). The same study revealed that AIP was able to interact with F-actin but in isolation has little biochemical effect upon the filament (there may have been some mild de-polymerisation evident). The co-addition of ADF however results in a dramatic synergistic increase in ADF's de-polymerisation activity and it has been shown that this is a result of AIP1 capping the barbed end of filaments (Okada *et al* 2002). In doing so AIP1 prevents the addition of monomers to the filament and so co-operates with ADF to induce the total de-polymerisation of F-actin.

Analysis of the completed *Arabidopsis* genome revealed two homologs of AtAIP1; one of which, i.e. AtAIP1-2 was constitutively expressed whilst the other AIP1-1 was confined to floral tissues (Allwood *et al* 2002). The same study utilised yeast two hybrid interactions to demonstrate that AtAIP1-1 was able to bind both G-actin and ADF *in vivo* and that it's addition to an *in vitro* co-

sedimentation assay increased the ability of a *Lillus* floral ADF to de-polymerise actin by 60%

Inducible down-regulation of AIP1 expression (via RNA interference) revealed that AIP1 was essential for the development of higher plants and that its role was effected via the regulation of Actin organisation (Ketelaar *et al* 2004). The absence of AIP resulted in a significant reduction in the growth of leaves, shoots flowers, roots and root-hairs; the severity of the phenotype corresponded with the degree of AIP knockout. Examination of actin distribution in the RNAi lines revealed (via expression of a GFP-Fimbrin Actin Binding Domain fusion construct) increased bundling in intercalary growing cells and an extension of bundles into the extreme apex of root hairs (Ketelaar *et al* 2004). It is suggested that this increase in actin bundling is a result of reduced filament disassembly, as the lack of AIP would allow increased re-addition of monomers to the barbed end of individual filaments.

The severity of the phenotype is surprising given that the knockout of AIP1 in C.elegans results solely in a loss of muscle contraction; the organism's development is otherwise unimpaired (Ono 2001). This serves as an illustration of the importance of correct actin organisation to plant development, a role that will be discussed more fully in section 1.3.2.

1.3 Functions and Roles of Actin.

The cytoskeleton plays an important role in the growth and development of higher plants and is also essential for the maintenance of house-keeping functions, such as endo and exocytosis, vesicle trafficking and cytokinesis (reviewed by Staiger *et al*, eds. 2000). An overview of some of these functions will be given within this section with particular attention being paid to topics relevant to this thesis. These include the role of actin in the growth and guidance of tip-growing cells , the role of actin in cellular morphogenesis and the importance of actin to cell expansion.

1.3.1 Cytoplasmic Streaming.

Cytoplasmic streaming is a key process which is essential for the transport of vesicles and organelles throughout the cell. This section can only

give a brief overview of what is both a fascinating and essential function of the actin cytoskeleton; interested readers are directed towards Grolig and Pierson (2000) in Staiger *et al* 2000 for further information.

The observation that regions of the cell exhibiting cytoplasmic streaming coincided with the staining patterns of actin filaments (Seagull *et al* 1987) and the discovery that the Cytochalasin-induced removal of F-Actin inhibited streaming (Bradley 1973) led to the belief that the actin cytoskeleton plays a key role in organelle movement and vesicle trafficking. Subsequent work with the lower plant *Chara* revealed that bundles of actin filaments provided a guideway for the movement of organelles (Kachar 1985).

Co-localisation of the actin-binding motor protein myosin with actin-filaments (Yokota *et al* 1999) and with motile organelles such as the Golgi Body (Nebenfuhr *et al* 1999) and the Peroxisome (Jedd and Chua 2002) suggests a role for mysosins as force-generators in cytoplasmic streaming. Three distinct families of myosin (VIII, XI and XIII) have been identified in higher plants with Myosin XI being the best characterised (reviewed in Shimmen and Yokota 2004). Both heavy chains posses an actin-binding N-terminal motor domain, which provides motive force for 'walking' along the actin-bundle at the expense of ATP, and a carboxyl-terminal domain that determines cargo selection. The interaction between myosin proteins and actin bundles is inhibited by the presence of Ca^{2+} and calcium-bound calmodulin (Yokota *et al* 1999). This provides a possible regulatory mechanism for cytoplasmic streaming in higher plants. In general myosins travel towards the barbed end of the actin filament, therefore the polarity of the filament determines the direction of travel. There does however appear to be an exception as pointed end directed Myosin VI family member has been characterised (Reviewed by Cramer 2000). The significance of this has yet to be determined.

The identification of actin comet tails (similar to those generated by *Listeria*) associated with motile organelles, such as phagosomes and endosomes (Southwick *et al* 2003) suggests that Arp2/3 induced actin nucleation and polymerisation may play a role in organelle movement. This is supported by the localisation of components of the Arp2/3 complex to the surface of mitochondria isolated from *S.cerevisae* and the inhibitory effect of Arp2/3 knockout upon mitochondrial movement (Boldogh *et al* 2001). It is interesting to note that the knockout of Arp2/3 complex does not affect the

localisation of the mitochondria to actin cables; this suggests that the Arp2/3 mediated nucleation and subsequent polymerisation play a role in force generation and therefore drive streaming (reviewed in Fehrenbacher et al 2003), but is not involved in the organelle/actin interaction

Migration of the yeast mitochondria differs from the Arp2/3-induced movement of *Listeria* in that preformed actin cables seem to be required to determine the direction of migration, i.e. the act as 'tracks' (Simon *et al* 1997). The mechanisms linking the newly formed force-providing 'cloud' of actin with the preformed guiding cables are poorly understood but it is believed that two mitochondrial membrane proteins Mmm1p and Mdm10p are responsible for a dynamic interaction between the organelle and F-actin (Boldogh *et al* 1998). It has been proposed that the comet tail actin is bound to the mitochondria in this way and that other actin-associated proteins incorporate this into the existing actin cables (Fehrenbacher *et al* 2003).

It has been suggested that cytoplasmic streaming is necessary as diffusion alone is not an efficient means of distribution in larger cells (Hochachka 1999). This is supported by the observation that the movement of peroxisomes was found to be considerably more significant in elongated cells (Jedd and Chua 2002) which has led to the suggestion that cytoplasmic streaming is used for long distance transport.

1.3.2 The Role of the Actin Cytoskeleton in Growth and Development.

The progression of a higher plant, such as Arabidopsis, from a seed to mature plant requires many processes but growth and development must be chief amongst them. Growth is often defined as an irreversible increase in volume and requires both cell division and cell expansion. Development involves the formation of specialised tissues and organs; ultimately this requires the differentiation, or morphogenesis, of individual cells. Most plant cells are bounded by a rigid cell wall that inhibits cell expansion and dramatic changes in shape as well as preventing cellular migration. So therefore changes in cell morphology require alterations to the cell wall. It is widely believed that the cytoskeleton plays a central role in both co-ordinating and mediating many of

these changes (Hussey *et al* 2004) and an overview of its involvement will be given here.

An increase in cell volume (i.e. growth) requires a corresponding increase cell wall and plasma membrane surface area, this is achieved by the addition of new material to both structures as there is limited capacity for stretching or thinning (Miller *et al* 1997). This new material consists of additional membrane packaged as vesicles, which is delivered via cytoplasmic streaming and added to the cell wall via exocytosis at the point of growth. Plant cells exhibit three distinct patterns of growth; Isodiametrical growth, Intercalary growth and Tip growth. Isodiametrical growth occurs across the entire surface of the cell and is driven by turgor pressure. Intercalary growth often results in the expansion of the cell along one axis (i.e. elongation) and is a result of membrane/cell wall expansion being confined to selected areas. Tip growth is highly localised and is the result of membrane/cell wall expansion being confined to one small area of the cell. An overview of these three forms of growth is given in Fig 1.6.

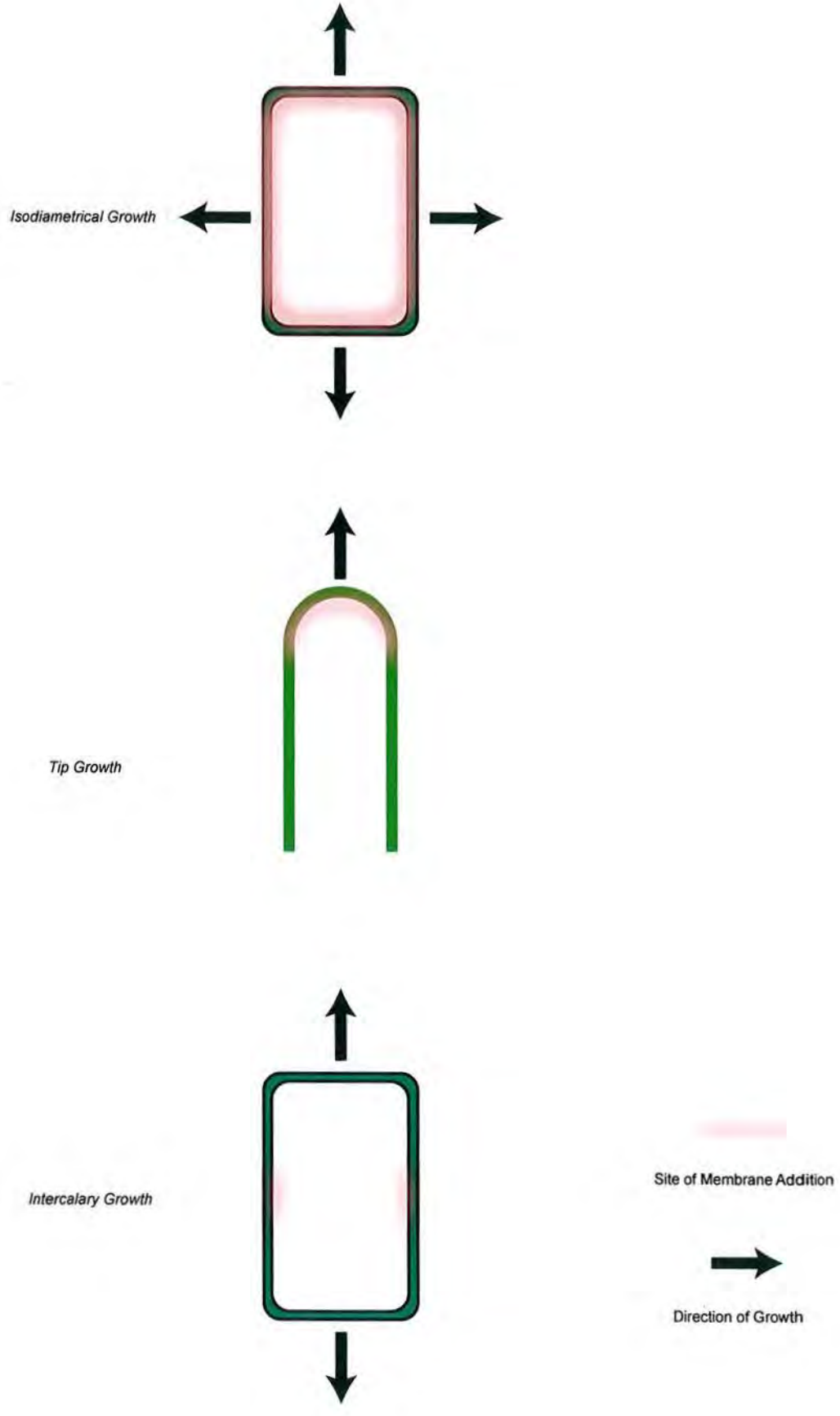
Cursory examination of a mature plant will reveal an enormous diversity of cell types, each with a morphology optimised to suit their physiological function, yet all originated from a single (mersistematic) cell type. Clearly plant cells must undergo a complex process of differentiation. It is significantly beyond the scope of this thesis to detail the biochemical changes with specialised cell types but morphogenesis as a whole shall be considered.

The exact shape of plant cells is often essential to their function, for example the colouration of petals is enhanced by the conical shape of their epidermal cells and lobed shape of leaf mesophyll cells increases the surface area of which gas exchange can occur (Smith 2003). Similarly the elongated form of the root hair increases the surface area of the root by upto 75% in some cases (Hussey *et al* 2004) and promote water uptake. The morphogenesis of the root hair will be considered in detail below.

1.3.3 The Root Hair as an Example of a Tip Growing Cell.

Tip growing cells such as root-hairs, pollen tubes and cultured xylogenic cells (Roberts and Uhnak 1998) have proven to be useful in the study of cell expansion as the exocytosis is highly localised and is amenable to manipulation

Figure 1.6: Forms of Plant Cell Expansion



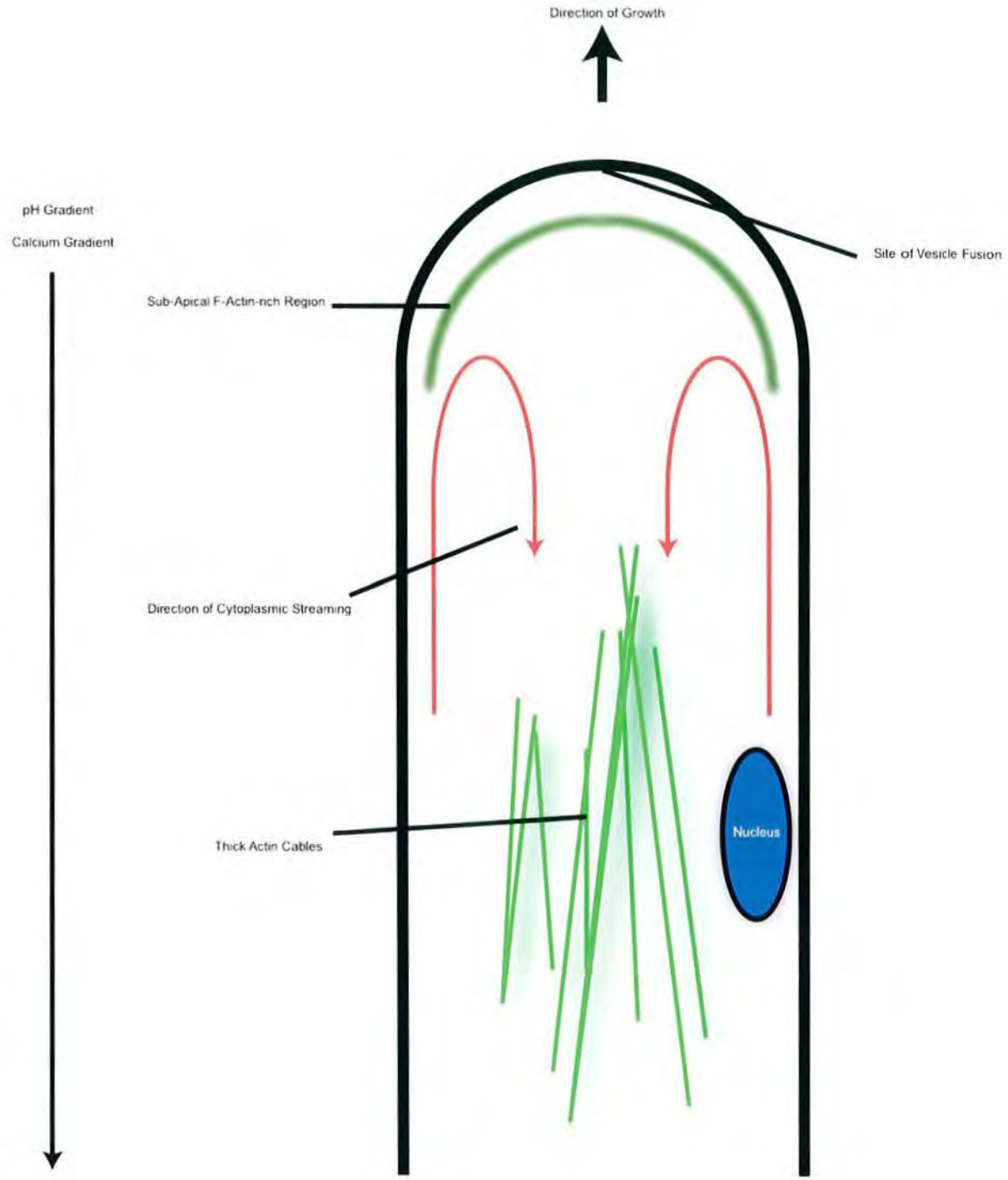
(comprehensively reviewed by Ketelaar 2002). The growing *Arabidopsis* root hair is an extension from a root epidermal cell and consists of a basal region in contact with the cell body, an elongated tube and an apical dome. This is shown diagrammatically in Fig 1.7.

Root hair growth begins with initiation, which in *Arabidopsis* consists of the formation of a bulge towards the base of a root epidermal cell. Bulge formation is marked by a local change of pH within the cell wall (Bibikova *et al* 1998) and by the accumulation of cell wall modifying enzymes such as Expansin (Baluska *et al* 2000). This would appear to be a cytoskeleton independent process as it is inhibited by neither Actin (Ovecka *et al* 2000) nor Microtubule (Ketelaar *et al* 2002) depolymerising drugs. There is however some support for the idea that actin plays a role in site selection as knockout of the *ACT2* gene results in irregular positioning (Ringli *et al* 2002). It also believed that the small GTPase Rop2 plays an important role as it has been shown to localise to the site of initiation and over-expression of a constitutively active mutant results in the formation of additional bulges (Jones *et al* 2002). There is some evidence that Rop's are responsible for the recruitment of actin to regions undergoing growth (Molendijk *et al* 2001) and so their localisation to the bulge may be a precursor to expansion.

The onset of root hair growth is marked by the establishment of a highly polarised cell. The extreme apex lacks both F-actin and organelles and is the site of exocytosis and membrane growth (Emons 1987), whereas the sub-apical region contains an extensive network of fine actin filaments that are believed to deliver vesicles to the tip. The root hair tube is packed with large villin-induced actin bundles surrounding a central vacuole (reviewed by Hussey *et al* 2004) and the cell gradually becomes more vacuolated towards the base. The position of the nucleus is maintained at a distance of approximately 75μM from the tip apex (in Arabidopsis) during growth (Ketalaar *et al* 2002). This precise positioning is actin dependant and it's disruption results in the migration of the nucleus towards the apex and an inhibition of hair growth. The mechanism of inhibition is not understood.

There is also a calcium gradient evident within the growing root hair cells, with the cytosolic concentration of Ca^{2+} ranging from 1μM at the apex to 200nM in the basal region (Fell and Hepler 1997). The influx of calcium to the apex is essential to growth (Schiefelbein *et al* 1993) and is itself dependent

Figure 1.7: An Overview of Tip Growth



upon the presence of Rop1 at the tip (Li *et al* 1999). Antibody staining has revealed Rops to be enriched at the apical tip (Molendijk *et al* 2001) and the fusion of Rop2 to Green Fluorescent Protein (GFP) has also suggested localisation to the tip (Jones *et al* 2002). The Stress Induced Mitogen Activated Protein Kinase (SIMK) represents another potential regulator of root hair expansion as it has also been shown to be enriched at the apical tip (Samaj *et al* 2002). The same study also revealed that the positioning and activation of the kinase were actin dependent and that it's inhibition leads to abnormal root hair growth. Its substrates however are still unknown.

Treatment of growing root hairs with the actin de-polymerising drug Cytochalasin D resulted in the loss of the sub-apical F-actin and a total inhibition of growth, although the thick bundles in the tube were not affected (Miller *et al* 1999). The application of a lower dose of Cytochalasin was found not to inhibit exocytosis at the tip but did result in a widening of the hair. This has been shown to be a result of exocytosis and therefore cell expansion occurring over a wider area (Ketelaar *et al* 2003). These results, and the observation that the knockout of the *ACT2* gene leads to an increase in root hair diameter (Ringli *et al* 2002), is suggestive of the sub-apical actin network confining the site of exocytosis to a small area of the tip. It is interesting to note that the over-expression of ADF in root hairs was also found to severely disrupt F-actin organisation and led to noticeably wider tubes (Dong *et al* 2001).

The addition of exogenous Oryzalin (a microtubule de-polymerising drug) does not affect the rate of cell expansion nor the diameter of the tube, suggesting that microtubules are not involved in the delivery of vesicles to the tip by cytoplasmic streaming and that they do not localise exocytosis (Ketelaar *et al* 2003). The same study however did reveal that microtubule de-polymerisation resulted in a loss of the growths strict directionality and could induce root hair branching; this is perhaps indicative of MT's regulating the position of the actin-defined exocytosis zone. The expression of an anti-sense α-tubulin gene also lead to root hair branching (Bao *et al* 2001), which again suggests a role for microtubules in determining root hair morphology.

It is believed that an outwards proton flux results in the sub-apical region exhibiting an alkaline pH (Hepler *et al* 2001), this has important ramifications for the regulation of the actin cytoskeleton as the interaction between many actin binding proteins and actin is pH regulated. An example would be ADF

(discussed in 1.2.5.2), which would expect to be highly active under physiologically alkaline conditions and may, via the severing induced generation of barbed ends, promote the formation of the fine sub-apical actin array.

The termination of root hair growth is marked by a loss of the calcium gradient (Wymer *et al* 1997) and the migration of the nucleus from its fixed position to a random site within the tube (Ketelaar *et al* 2002). In addition, the cytoplasmic organisation of the apex will alter to reflect that of the tube, i.e. the cytoplasm becomes less dense and vacuoles and other organelles are no longer excluded.

1.3.4 Pollen Tubes as Tip Growing Cells

The pollen tube is an example of another cell type that demonstrates both dramatic elongation and tip growth. They are responsible for the transport of the male gametes to the Oocyte and may extend to a length of several centimetres. Pollen tubes are in many ways similar to root hairs in their manner of growth and cytoskeletal organisation but there are a few key differences. It appears that fine sub-apical bundles do extend to the extreme apex in pollen tubes and there is relatively little cellulose at the growth tip (reviewed in Smith 2003). It is believed that Rop1 and Rop5 are localised to the apical tip and that they actively promote the recruitment of Actin (Li *et al* 1999). In addition, there is some evidence that the direction of pollen tube growth responds to external signalling stimuli such as a gradient of cAMP (Moutinho *et al* 2001). This provides a mechanism by which pollen tubes could be accurately guided to the stigma.

1.3.5 The Expansion of Intercalary Growing Cells

Intercalary growth is most often described as the turgor pressure driven expansion of a cell along a single axis, this may also be defined as anisotropic growth and is typified by cell elongation (reviewed by Smith 2003). The direction of such expansion is usually transverse to the orientation of cellulose microfibrils within the cell wall, which have been said to act as if they were 'hoops around a barrel' in such cells and so oppose radial expansion (Mathur

and Hulskamp 2002). The orientation of cellulose microfbrils usually mirrors that of the underlying microtubules and so regulation of the cytoskeleton is likely to be critical in controlling Intercalary growth. Evidence for this comes from the isotropic cell expansion observed when cells are treated with microtubule de-polymerising drugs (discussed in Wymer and Lloyd 1996). So in effect the orientation of microtubule deposition determines the direction in which the cell may expand.

The role of actin in Intercalary growth is less clear although a diffuse network of fine actin networks has been observed at the point of cell expansion (Fu *et al* 2002) and treatment with Actin depolymerising drugs inhibits cell elongation (Baluska *et al* 2000). Additionally a reduction in the expression of the actin interacting protein AtAIP1-1 was shown to result in actin bundles of increased thickness and a decrease in cell expansion (Ketelaar *et al* 2004). It is believed that the Rop family of small GTPases play a key role in regulating actin as the expression of a constitutively active Rop2 mutant induces isotropic expansion and the expression of a dominant negative mutant reduces both the amount of visible F-actin and the degree of cell expansion (reviewed by Hussey *et al* 2004).

1.3.6 The Cytoskeleton in Higher Plant Morphogenesis.

The rigidity of the plant cell wall means that cell division must be subjected to strict control, with both the number of divisions and the plane of division being tightly regulated. Cell division results in two daughter cells that are separated by a newly formed cell wall, which will have developed from a cell plate situated between the daughter nuclei (reviewed by Kost *et al* 1999). The cell plate is initiated in the centre of the cell and will grow outwards in a centrifugal manner until it has fused with the parental cell wall. It is believed that a microtubule and actin comprised structure known as the pre-prophase band (PPB) demarcates the site where the cell plate and cell wall shall fuse, which suggests a regulatory role for the cytoskeleton in this process. Further evidence stems from the detection of short actin filaments at the edge of the growing cell plate (Endle *et al* 1998) and the observation that treatment with actin depolymerising drugs leads to abnormal cell plate positioning and therefore to aberrant cell divisions (Mineyuki and Palevitz 1990).

1.4 The Role of Cyclic Adenosine Monophosphate (cAMP) in Higher Plants.

1.4.1 An Introduction to cAMP signalling

The study of the signalling pathways linking the hormone Adrenaline to the metabolic enzyme Glycogen phosphorylase led to the discovery of adenosine 3'5'-cyclic monophosphate (cAMP) (Rall *et al* 1957), an important secondary messenger molecule that is conserved across kingdoms. A model for cAMP signalling was originally proposed by Robinson and Sutherland in 1971 and a modified form is shown in Fig 1.8; in essence it is believed that the binding of a primary messenger (such as a hormone) to a seven trans-membrane domain receptor protein results in the disassociation of the α-sub-unit of an intra-cellular GTP-bound protein (a G-protein). This in turn prompts the activation of an intracellular membrane-bound Adenylate Cyclase which catalyses the production of 3'5'cAMP from an ATP substrate. The cAMP will then diffuse throughout the cell and interact with downstream proteins before it is hydrolysed to the inactive 3' form. Activation of the Adenylate Cyclase will cease when the G-proteins α-subunit hydrolyses its bound molecule of GTP and disassociates from the enzyme.

There are several down-stream effectors of cAMP with the cAMP-dependent Protein Kinase (PKA) being one of the most important. The binding of two molecules of cAMP to PKA's regulatory subunit results in its disassociation from the catalytic subunit and the subsequent activation of PKA. The enzyme will then phosphorylate a wide variety of proteins (including many enzymes) and in doing so will induce a change in their activities. In addition PKA can also permit regulation of gene expression to be linked to cAMP signalling as it has been shown to phosphorylate the cAMP response element (CREB), a transcription factor. The phosphorylation of CREB by PKA induces dimerisation and association with two other proteins; the CREB binding protein (CBP) and p300. This complex is then able to promote the transcription of genes containing a CREB response element (CRE) sequence in their promoter (reviewed in Newton and Smith 2004). Several proteins show a direct response to cAMP; Bradley *et al* (1994) identified a cAMP regulated K^+ channel and a Guanine exchange factor (GEF) called Epac has been shown to be directly activated by cAMP (De Rooj *et al* 1998). This particular GEF is believed to

Figure 1.8: The cAMP Signalling Pathways of Higher Eukaryotes.

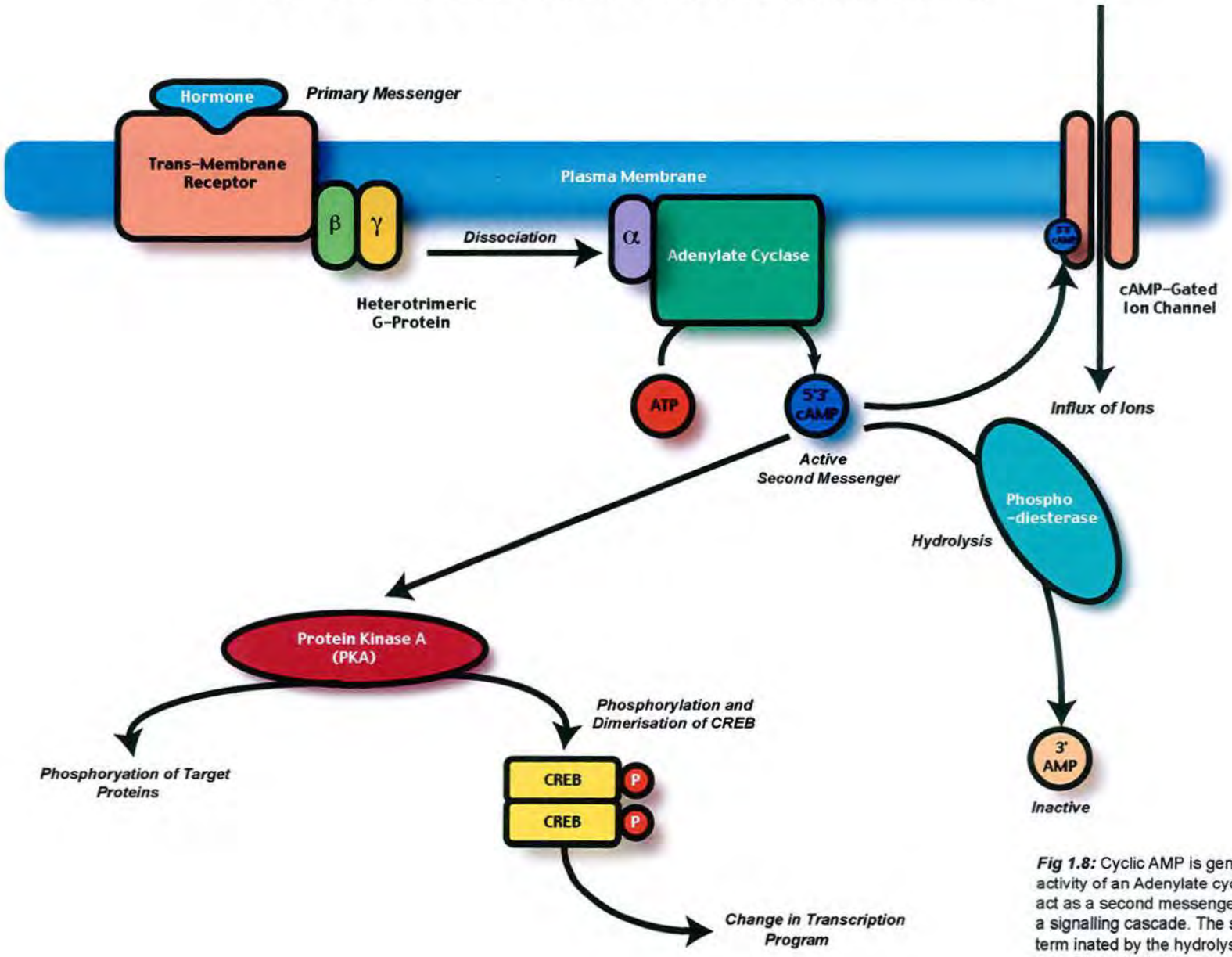


Fig 1.8: Cyclic AMP is generated by the activity of an Adenylate cyclase and will act as a second messenger by initiating a signalling cascade. The signal will be term inated by the hydrolysis of cAMP by a Phosphodiesterase.

induce the release of GDP by the Rap 1 GTPase and in doing so promote GTP uptake by the Rap and therefore its activation. It can therefore be seen that cAMP is a important signalling molecule that is able to promote a diverse array of downstream responses and is itself, through a variety of G-Protein coupled receptors, responsive to multiple inputs. In this way it is able to cross-link many signal transduction pathways.

1.4.2 The Evidence for cAMP Signalling in Higher Plants

The role of cyclic AMP or even its existence in higher plants has proven controversial due to both the small quantities found and the (to date) absence of a candidate Adenylate Cyclase. Mammalian tissue will contain approximately 100-500 pmol g^{-1} of cAMP, whereas the concentration found in higher plant tissue is much lower, (5 pmol g^{-1} from cultured *Phaseolus vulgaris* cells) (Bolwell 1995). This suggests that the concentration of cAMP is too low to act as a secondary messenger but there are several plant signalling molecules, such as phospholipids, than have been shown to be effective at pM concentrations. In addition the measured values are averages for an entire tissue sample, local concentrations within the cell may be significantly higher, allowing localised responses to be induced. In addition, it is entirely possible that the concentration may rise in response to environmental stimuli such as pathogen attack; many of the closest matches for Adenylate cyclases in the *Arabidopsis* genome have been identified as disease resistance proteins. So the low concentration of cyclic AMP need not mean that it does not play a functional role in the plant cell.

The use of mass spectroscopy has allowed the concentration of cAMP in tissues to measured and compared and it has shown that plasma membrane preparations from Phaseolus vulgaris have the ability to synthesise cAMP, i.e. there is *de novo* cAMP synthesis thereby implying that there is a membrane bound Adenylate Cyclase present (Roef *et al* 1996). Similar activity has been reported in the chloroplasts of *Nicotiana tabacum* (Witters *et al* 2004) and *S.oleracea* (Newton *et al* 1999). Interestingly it appears as if the *N.tabacum* enzyme is G-protein linked as its activity was increased by the addition of exogenous GTP and it was inhibited by 5'-O-(2-thiodiphosphate), a potent G-

protein inhibitor. In addition it is also inhibited by the non-hydrolysisable cAMP analogue dideoxyadenosine but in contrast to mammalian Adenylate cyclases was found not stimulated by Forskolin.

The synthesis of cAMP is only one component of the signalling pathway as the messenger must also have targets to act upon and a mechanism by which it may be degraded. Several cAMP-dependant protein kinases have been identified from a wide variety of plants including *Zea mays* (Janistyn 1972), *Oryza sativa* (Lawton *et al* 1989) and *Arabidopsis* (Hayashida *et al* 1993). There is also evidence of the CREB pathway being present in higher plants as a close homolog of CREB has been isolated from *Vicia faba* (Ehrlich *et al* 1992). It is also believed that a plant cAMP-dependant protein kinase (PKV from *Lycopersicum esculentum*) contains a nuclear targeting sequence and so would be localised to the nucleus and would therefore be in a position to phosphorylate CREB (Hammond and Zhao 2000).

It is believed that there is a relatively large group (approximately 20 distinct genes in *Arabidopsis*) of plant cAMP-gated ion channels that have been shown to be opened solely when cyclic nucleotides are bound (reviewed in Talke *et al* 2003) implying that cAMP in plants may have an effect beyond stimulating kinases. Interestingly activation of some of these channels has been shown to be reversed by the binding of Ca^{2+} (Hua *et al* 2003) and so it is likely, that in this case, Ca^{2+}, cAMP, and their respective signalling pathways have antagonistic effects.

The information presented above shows that many of the constituent proteins and pathways required for cAMP signalling are present in higher plants, yet the work of Moutinho e*t al* (2001) is to date the only reliable in depth study of the developmental role played by cAMP signalling. This study found that germinating pollen tubes grew towards a gradient of cAMP and the AC activator forskolin but grew away from a gradient of the AC agonists dideoxyadenosine and Theophylline. The addition of exogenous forskolin and cAMP was found to dramatically increase in the intra-cellular concentration of cAMP whereas the application of dideoxyadenosine decreased it. These data suggested that not only is pollen tube growth responsive to cAMP but also that the tubes themselves express an adenylate cyclase with functional similarities to the mammalian enzymes. In addition, Moutinho *et al* (2002) identified PsiP, a

candidate andenylate cyclase from *Zea mays* and showed that it's down-regulation significantly altered tip morphology in growing tubes.

1.5 Cyclase Associated Proteins.

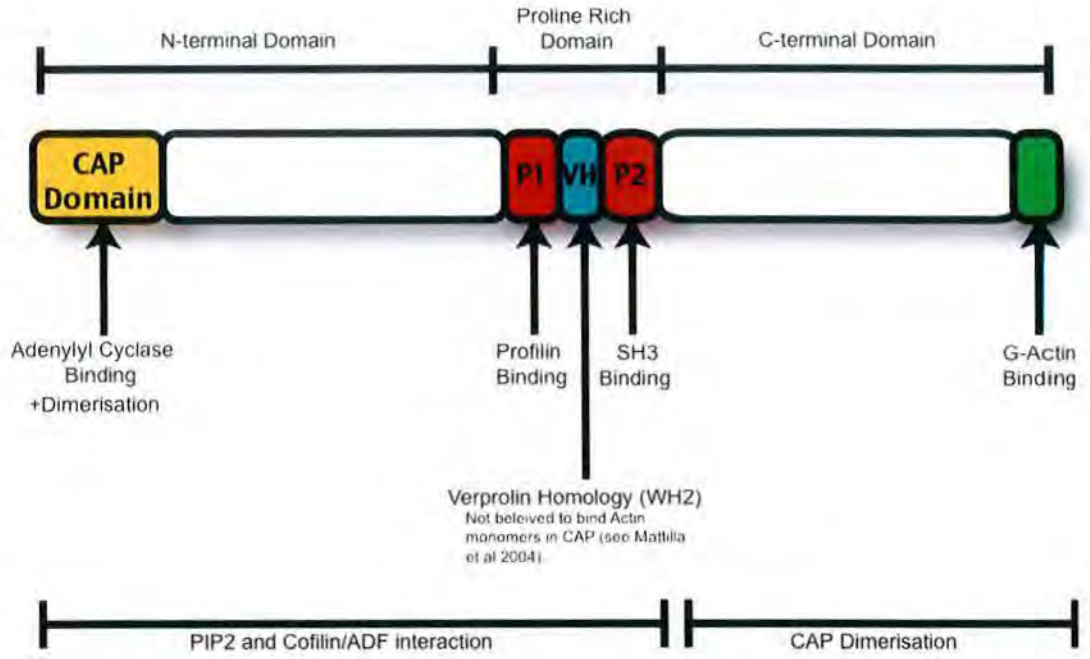
The Cyclase associated protein (CAP) family was initially identified in the budding yeast *Saccharomyces cerevisiae*, but is believed to be ubiquitous throughout the *Eukaryote* (reviewed in Hubberstey and Mottillo 2002). The majority of organisms examined to date (including all higher plants) possess a single CAP isoform although it appears mammalian genomes contain two CAP isoforms with distinct expression patterns (Swiston *et al* 1995). CAP is believed to be a bi-functional protein as it has, in yeast, been shown to stimulate adenylate cyclases and is also thought to be an important regulator of actin dynamics. Several conserved domains have been identified throughout the CAP family. These include an amino adenylate cyclase binding domain, a central Profilin-binding poly-proline domain and a carboxyl actin interacting domain (Stevenson and Theurkauf 2000). An overview of these domains is given in Fig 1.9 and a description of their roles in CAP function is included within the next few sections.

There is a reasonable amount of evidence that CAP may form higher order structures, such as dimers, trimers and multimers although the functional significance of this is not yet clear (Yang *et al* 1999); a fuller description of CAPs tendency to self-associate is given in section 1.5.3. The structure of a full length CAP is yet to be determined but diffraction studies upon the N-terminus of *Dictyostelium* CAP have shown it to be comprised almost entirely of bundled α-helices arranged into six anti-parallel strands (Ksiazek *et al* 2003). Interestingly an associated Mg^{2+} ion seemed to be critical for CAP N-terminal dimerisation.

1.5.1 The Identification of CAP and Involvement in cAMP signalling.

CAP was initially isolated as a 70KDa component of the *Saccharomyces* CYR1 adenylate cyclase complex and was found to be required for the enzymes activity (Field *et al* 1990). Elimination of CAP expression was found to result in a number of distinct phenotypes including abnormal morphology

Fig 1.9: Domain Structure of the *S.cerevisiae* CAP Potein



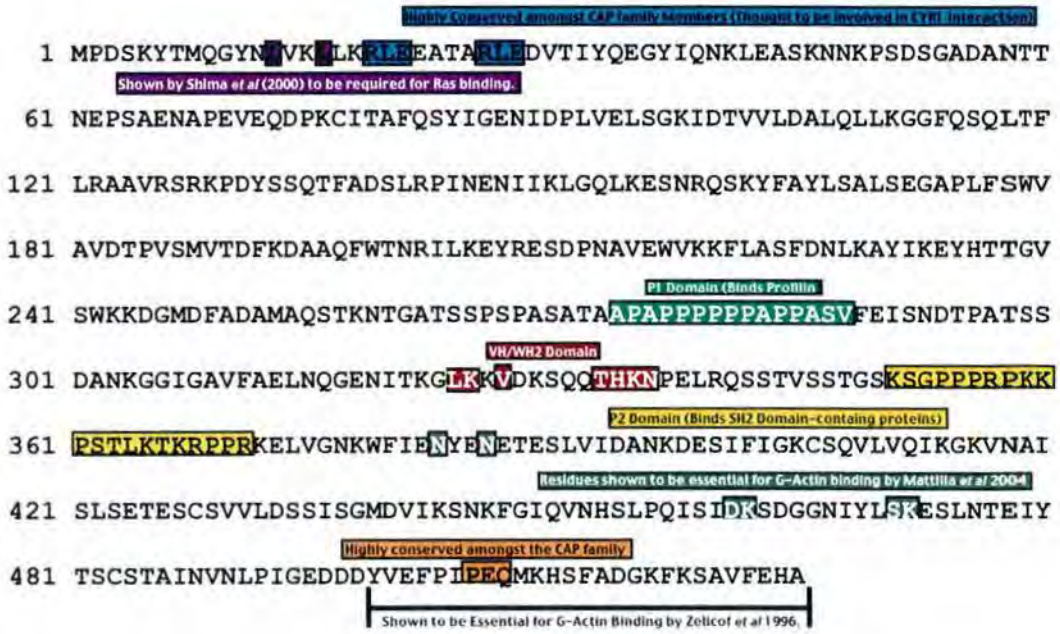


Fig 1.9: The general domain structure of CAP is shown above with the general attributes of each domain labelled. A map showing residues of interest is given below.

(enlarged and unusually round cells), temperature sensitivity, increased sensitivity to nitrogen starvation and an inability to grow on rich media. All of these characteristics were suppressed by complementation with the Cap gene.

The CYR1 adenylate cyclase is known to be stimulated by the small GTP binding protein RAS2 and it has been shown that the substitution of a Glycine residue with Valine at position 19 in Ras2 leads to a reduction in Ras's intrinsic GTPase activity and therefore over-stimulation of CYR1. The result of this mutation is a cellular accumulation of cAMP and a subsequent inability to grow on media containing galactose (Deschenes and Broach 1987) coupled with increased resistance to heat-shock. An ethylmethanesulphonate (EMS) screen of this $RAS2^{Val19}$ background identified genes that were able to restore the ability of the yeast to grow upon galactose; several of the revertant clones were found to contain a mutated form of the *cap* gene that was designated *SRV1* (for Suppressor of $Ras2^{Val19}$) (Fedor-Chaiken *et al* 1990). The *SRV* mutation was found to severely reduce (although not eliminate) production of cAMP, suggesting that CAP plays a key role in the activation of CYR1 by Ras. Deletion studies suggest that the N-terminal domains of CAP are responsible for the interaction with and activation of CYR1 as expression of the 168 amino-terminal residues of CAP was sufficient to induce cAMP production, whereas the carboxyl-terminal residues (numbers 369-526) had no effect (Shima *et al* 1997). Further studies have since demonstrated that the interaction between CAP and CYR1 is dependant upon the first 36 residues of CAP (Nishida *et al* 1998).

The CYR adenylate cyclase contains a central Leucine rich domain which preferentially interacts with GTP-bound Ras, whereas a complex of CAP and the C-terminal fragment of CYR (residues 1764-2026) seems to produce a second binding site for farnesylated Ras that is not dependant upon the bound nucleotide (Shima *et al* 2000). Neither the C-terminal fragment of CYR1 nor CAP were able to bind Ras in isolation, suggesting that the second binding site is a product of both proteins. The same study showed that mutagenesis of the CAP adenylate cyclase binding domain could produce proteins that had lost the ability to stimulate cAMP production but had retained capacity to interact with CYR1. These mutant CAPs were shown to have either lost the ability to interact with Ras completely or to have a significantly increased affinity for it. So it can be seen that the interaction between CAP and Ras is as critical as the

interaction between CAP and CYR in regulating Ras-mediated cAMP production.

Yeast are thought to be unique in that the production of cAMP is stimulated via the Ras signalling pathway; in most organisms this is activated as detailed in 1.4.1 (i.e. by the association of the α-subunit of a hetrotrimeric G protein with an adenylate cyclase). This raises important questions regarding the requirement for CAP in the production of cAMP within higher Eukaryotes. The discovery that the CAP of *Cryptococcus neoformans* (a pathogenic fungus) is able to bind the Gα subunit Cac1 (via the sub-units C-terminus) and was essential for the production of cAMP suggests involvement in the alternate pathway (Bahn *et al* 2004). It is entirely possible that higher eukaryotic CAP's facilitate the interaction between Gα proteins and adenylate cyclases in a manner similar to the CAP-mediated interactions between adenylate cyclases and Ras proteins in lower Eukaryotes.

It is important to note that a direct physical interaction with adenylate cyclases has only been demonstrated for the CAP's of *Saccharomyces cerevisiae* (Field *et al* 1990) *and Schizosaccharomyces pombe* (Gerst *et al* 1991). There is however evidence that CAP participates in the cAMP signalling pathways of other organisms as deletion of the CAP gene in the pathogenic fungus *Candida albicans* prevented the formation of germ tubes and virulence (Bahn and Sundstrom 2001). The phenotype was rescued by the addition of exogenous cAMP. The slime mould *Dictyostelium discoideum* is known to respond to extra-cellular cAMP gradients both by developing cellular polarity and by producing and secreting further cAMP (Firtel and Chung 2000). A severe reduction of CAP expression has been shown to reduce the degree of polarisation exhibited in response to a cAMP gradient and to significantly curtail the production of additional cAMP (Noegel *et al* 2004). This suggests that CAP plays a role in both cAMP sensing pathways and the production of cAMP within *Dictyostelium* but it is important to note that the same study failed to show a direct interaction between CAP and an adenylate cyclase via a yeast two hybrid experiment.

1.5.2 Interaction with the Actin Cytoskeleton

Fig 1.10: Two Models for the Activation of Adenylate Cyclase by CAP in Yeast and Higher Eukaryotes.

Yeast Model

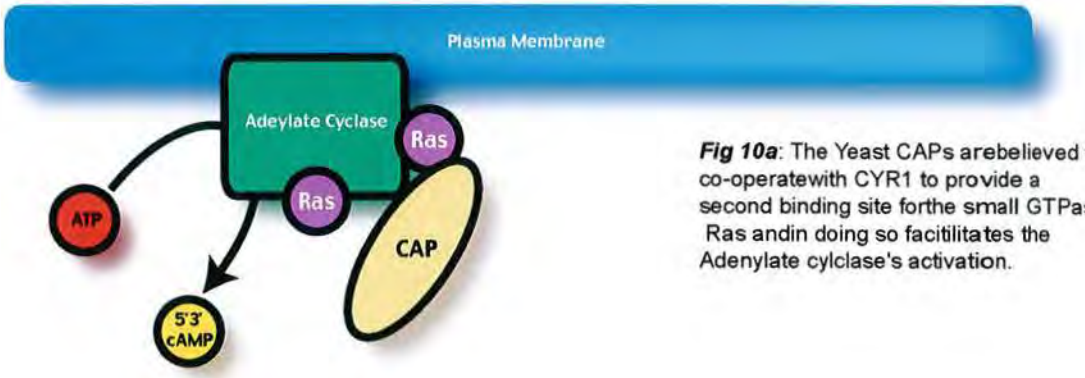


Fig 10a: The Yeast CAPs arebelieved to co-operatewith CYR1 to provide a second binding site forthe small GTPase Ras andin doing so facitilitates the Adenylate cylclase's activation.

Higher Eukaryotic Model

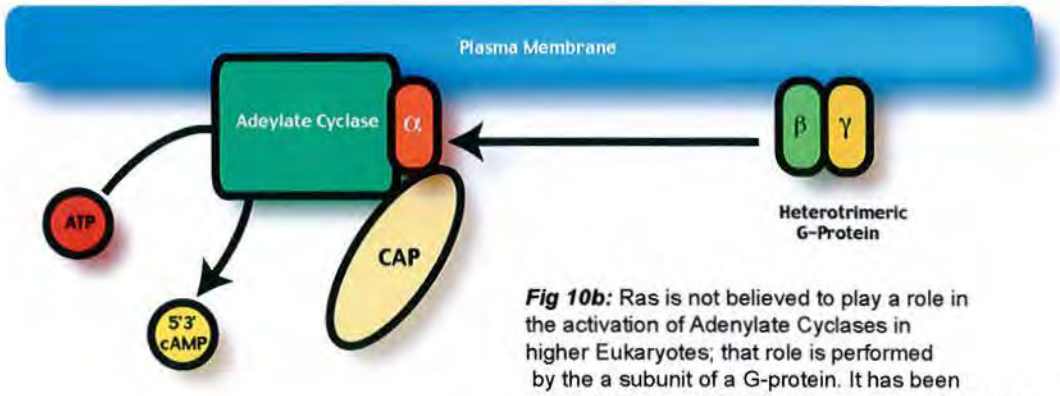


Fig 10b: Ras is not believed to play a role in the activation of Adenylate Cyclases in higher Eukaryotes; that role is performed by the a subunit of a G-protein. It has been shown that the CAP o*f Cryptococcus neoformans* may bind both the AC andthe a subunit and in doing so stimulate cAMP production.

The complementation of Ras-deficient *S.cerevisiae* with an active cAMP-dependant protein kinase results in the suppression of loss of Ras phenotypes but the addition of the same construct to CAP-deficient cells did not result in complete complementation of the phenotype (Field *et al* 1990). In particular the complemented cells still exhibited an abnormal morphology and an inability to grow upon rich media. This suggests that at least some of CAPs functions are independent of the Ras/cAMP pathway and the morphological alterations are indicative of a cytoskeletal defect. Examination of the actin cytoskeleton of the CAP mutants revealed a severely disrupted actin cytoskeleton where actin cables were entirely absent and the polarity of actin depositition had been lost (Vojtek *et al* 1991). The observation that over-expression of the actin monomer sequestering protein Profilin II (see 1.2.5.2) in CAP-deficient yeast restores wild-type morphology and alleviates sensitivity to nitrogen starvation (Vojtek *et al* 1991) provides further evidence in support of an additional actin-related role for CAP and suggests a functional relationship between CAP and Profilin (Goldschmidt-Clermont and Janmey 1991). It also interesting to note that the knockout of both Profilin I and II homologs in the slime mould *Dictyostelium discoideum* results in 73% increase in cellular concentration of CAP (Gottwald *et al* 1996) which again suggests some functional redundancy between the proteins.

Interestingly complementation with Profilin I did not restore the wild-type phenotype despite both isoforms playing a similar role in the regulation of actin dynamics (Vojtek *et al* 1991). The major functional difference between yeast Profilins I and II is the significantly higher binding affinity for PIP_2 demonstrated by Profilin II (Machesky *et al* 1990). As a result it has been proposed that the rescue of CAP mutants by Profilin II is a result of the restoration of correct poly-phosphoinositide signalling as opposed to redundancy in actin modulating functions between CAP and Profilin (Goldschmidt-Clermont and Janmey 1991). There is however overwhelming evidence that CAP plays a key role in the regulation of actin and this will be discussed fully within the following paragraphs.

The morphological and nutrient sensitive phenotypes were rescued by the restoration of the carboxyl-terminus (the final 237 amino-acid residues) of CAP, whereas complementation of the mutant with the Ras/CYR1-interacting N-terminus had no effect (Gerst *et al* 1991). So it can be concluded that CAP is,

at the very least, a bi-functional protein and distinct functions are performed by the amino and carboxyl termini. Immuno-precipitation experiments have revealed that *S.cerevisiae* CAP bound G-actin with a 1:1 stoichiometry (Freeman *et al* 1995) and this function has been localised to the carboxyl terminus via *in vitro* experiments with expressed truncated CAP fragments and was independent of the N-terminal and proline rich regions (Zelicof *et al* 1996). The addition of CAP to *in vitro* actin polymerisation assays resulted in a significantly reduced rate of polymerisation which is indicative of a monomer sequestering function; again this was dependant upon the carboxyl terminal. These results constitute strong evidence in support of CAPs carboxyl-terminal functions being related to the regulation of the actin cytoskeleton.

The complementation of CAP-deficient yeast with truncated CAP genes revealed that a construct lacking codons representing the last 27 amino-acids were unable to complement the carboxyl-terminus associated phenotypes (Zelicof *et al* 1996). Further results from the same study showed that, when expressed, this protein was unable to bind actin monomers. This permits more specific localisation of the G-actin binding site (to the extreme carboxyl terminus) and more importantly that the phenotypes are linked to an inability to bind actin. Comparison of the sequences of CAP family members reveals several short motifs within this region that are highly conserved across the entire family (Hubberstay and Mottillo 2002); these residues are highlighted in Fig 1.9. The substitution of charged amino-acids throughout the carboxyl terminus with uncharged Alanine residues has been used as a tool to identify the specific motifs responsible for G-actin binding (Mattilla *et al* 2004). Proteins with alterations at three sites (i.e. residues 383+386, 461+462 and 472+473) were found to be unable to complement the mutants in vivo studies and to lack the ability to bind monomeric G-actin in *in vitro* assays. It is important to note that these three site all reside outside the final 27 amino acids and so it is likely that CAP/Actin binding is also dependant upon interactions between uncharged residues. Previously it had been suggested that a candidate WH2-like domain that had been identified adjacent to the proline-rich region may be responsible for actin-binding as WH2 domains are able to bind actin in VASP family members (Baum *et al* 2000). Mutagenesis of the candidate motif did not however alter CAP's measure affinity for G-actin in *in vitro* binding assays and so this possibility may be discounted (Mattilla *et al* 2004). The sites described

above are also highlighted upon the diagram of S.cerevisiae CAP shown in Fig 1.9

Characterisation of the interaction between G-actin and CAP has given some insight into the effect of CAP upon actin dynamics. A thorough study by Gottwald *et al* (1996) revealed that at a 1:1 molar ratio *Dictyostelium* CAP was able to completely inhibit the formation of F-actin and that the binding of PIP_2 to CAP was able to prevent both its binding to actin and its ability to prevent polymerisation. From this it could be concluded that CAP is a phospho-inosotide responsive actin sequestering protein. Interestingly the site of PIP_2 binding was shown to lie outside the carboxyl-terminus as the in vitro activity of this region alone was no responsive to PIP_2 although the precise binding site has not been identified.

The interaction of CAP with other actin-modulating proteins and the effect of those interactions upon actin dynamics reveals gives further insight into the physiological role of CAP. *In vitro* binding experiments revealed that Human CAP1 was able to associate with the Cofilin-Actin complex and further experiments demonstrated that CAP was able to accelerate the rate of Cofilin-induced actin de-polymerisation at the pointed end of the filament (Moriyama and Yahara 2002). Interestingly more detailed experiments revealed a concentration dependant bi-phasic effect where CAP (in the presence of cofilin) upto a concentration of 1μM was able to promote the net addition of monomers to the barbed end of the filament. The addition of CAP in excess of this concentration resulted in a net depolymerisation and the balance between the two effects was dependant upon the presence of cofilin; in it's absence CAP prompted de-polymerisation at a much lower concentration (0.2μM). The polymerisation was attributed to the barbed end as CAP was unable to promote the addition of monomers to Gelsolin capped filaments (Moriyama and Yahara 2002). There is however some debate about this affect as separate study failed to demonstrate that CAP, at any concentration, was able to directly promote polymerisation (Balcer *et al* 2003).

In vitro biochemical studies have been used to show that CAP binds ADP-associated G-actin with a significantly higher affinity than it does the ATP-associated monomer. This suggests that CAP may, at low concentrations, promote actin dynamics by recycling the monomer. The fact that CAP and Cofilin compete for the same binding site upon actin (Mattila *et al* 2004) permits

Figure 1.11: Models for the CAP-Mediated Regulation of Actin Dynamics in Yeast

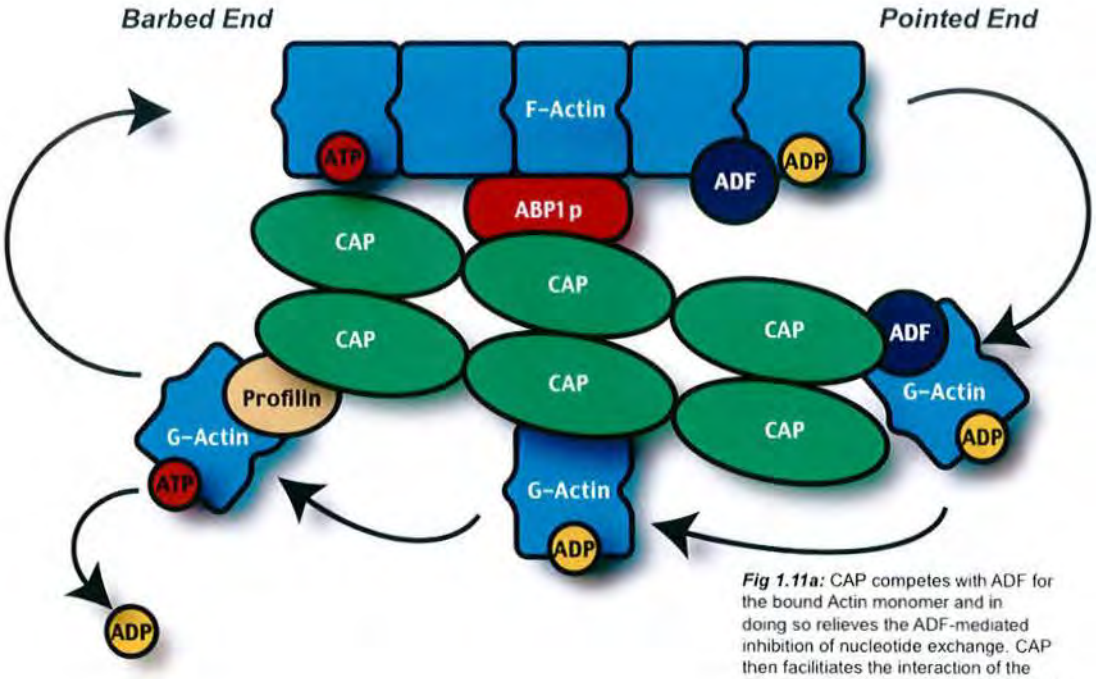


Fig 1.11a: CAP competes with ADF for the bound Actin monomer and in doing so relieves the ADF-mediated inhibition of nucleotide exchange. CAP then facilitiates the interaction of the monomer with Profilin thereby promoting nucleotide exchange and polymerisation at the barbed end. The regeneration of ADF will also be accelerated.

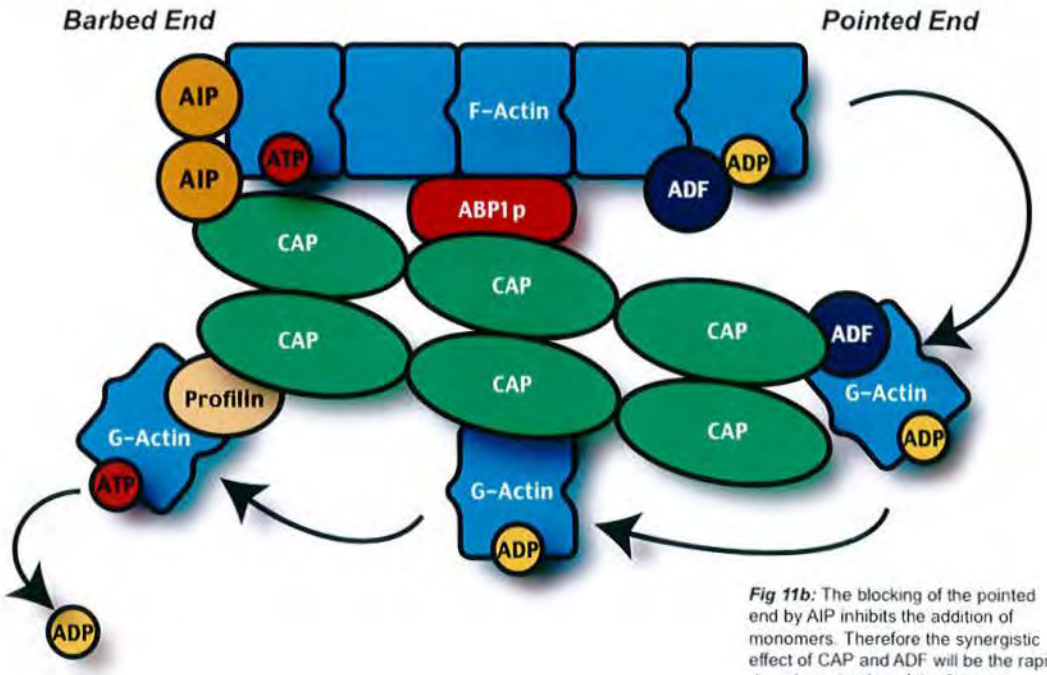


Fig 11b: The blocking of the pointed end by AIP inhibits the addition of monomers. Therefore the synergistic effect of CAP and ADF will be the rapid de-polymerisation of the filament. The localisation of AIP to the pointed end may be facilitated via its interaction with CAP.

a general model of CAPs role in the modulation of actin dynamics to be constructed; this is shown in Fig 1.11 In essence it is believed that CAP directly competes with Cofilin/ADF family members for the ADP-bound G-actin that they have removed from the pointed end of the actin filament. In liberating the monomer from cofilin CAP relieves the inhibition of actin nucleotide exchange induced by cofilin and in doing so will promote the re-generation of ATP-bound actin. The end-result of this is an increase in the pool of available polymerisable actin monomers and the regeneration of free cofilin, both of these factors will contribute to an increase in actin dynamics. It is reasonable to propose that Profilin will be involved in this process as the addition of Profilin to an *in vitro* polymerisation experiment containing CAP in excess of its inhibitory concentration was able to partially relieve the blocking of monomer addition to the barbed end (Mattila *et al* 2004). This has led to the suggestion that CAP hands-off its bound monomer to Profilin, which in turn will promote nucleotide exchange and therefore addition to the barbed end of the filament as described in 1.2.5.2. This is unlikely to occur in plant cells as Plant profilin homologs have been shown to act solely as monomer sequestering proteins and do not promote polymerisation or nucleotide exchange. The role of CAP in the plant system is therefore unclear.

The idea that CAP promotes actin filament dynamics is supported by some *in vivo* evidence as video-microscopy of CAP-deficient human cells shows that F-actin structures showed less re-organisation with the end result being an inhibition of migration (Bertling *et al* 2004). The same study used Fluorescence Recovery After Photo-bleaching (FRAP) techniques to demonstrate that the turnover of monomers within stress-fibres was inhibited by the absence of CAP, as the fluorescence of bleached sections of fibre did not recover in CAP-cells. Finally the addition of the monomer sequestering drug Latrunculin A resulted in significantly less depolymerisation on CAP deficient cells than it did in the wild type, which is indicative of lower turn-over rates as the drug is unable to directly induce de-polymerisation.

1.5.3 The Regulation of CAP, its Dimerisation and Interactions with Other Proteins

The complex nature of CAP-function would imply an equally complex mechanism of regulation and localisation, so as to permit spatial and temporal control. There is a growing body of evidence that expression of CAP is confined to certain tissues at specific stages of animal development (see 1.5.5) and that this transcriptional control provides a primary method of regulation.

The localisation of a protein has a profound effect upon its activity by determining which other proteins and structures with which it will come into contact. Analysis of the primary structure of *S.cerevisiae* CAP reveals two separate proline-rich regions towards the centre of the protein, which were designated P1 and P2, with the one closer to the carboxyl-terminus (i.e. P2) matching the consensus sequence (i.e. PPxPPPxP) for a SH3 recognition site (Hubberstey and Mottillo 2002). The Src homology 3 (SH3) domain is a small motif with a high affinity for poly-proline domains; and is believed to mediate protein-protein interactions. (Pawson and Schelessinger 1993). When expressed full length *S.cerevisiae* CAP has been shown to be able to interact with the SH3 domain of ABP1p, a F-actin binding protein (Kessels *et al* 2000), in *vitro* and the subsequent expression of a truncated fragment allowed this interaction to be localised to CAP's P2 domain (Freeman *et al* 1996). This binding of CAP to the expressed SH3 fragment did not affect its interaction with either monomeric actin of the CYR1 adenylate cyclase.

The same study demonstrated that the deletion of the poly-proline domain disrupted the *in vivo* localisation with cortical actin patches in yeast whereas deletion of the C-terminus and therefore the G-Actin binding region did not. It can therefore be stated that the localisation of CAP to F-actin in yeast is independent of its interaction with monomeric actin. There is strong evidence suggesting that ABP1p is the CAP-localising factor as it's deletion also results in the loss of CAP from cortical F-actin patches in *S.cerevisiae* (Lila and Drubin 1997). This appears to be a mechanism specific to CAP as the deletion of ABP1p did not affect the localisation of other cortical actin binding proteins such as Cofilin or Sla1p. The interaction of CAP with ABP1p, and therefore its localisation, appears to be functionally independent from it's roles in cAMP signalling and general actin modulation as truncated CAP's lacking the poly-proline domain are capable of fully complementing the *Srv2* phenotype (Lila and Drubin 1997). Higher plants lack a homolog to ABP1p (Hussey *et al* 2002) so it is unlikely that plant CAP is localised to F-actin in this manner. It is possible that

either plant CAP's do not associate with F-actin, that a different bridging protein is involved or that the interaction was direct.

Further experiments have suggested that the localisation of CAP to F-actin is more complex than the aforementioned experiments would suggest as mutations within the adenylate cyclase binding region (at the N-terminus) also prevents localisation to cortical actin patches (Yu *et al* 1999). These specific N-terminal mutations (shown in Fig 1.9) did not however affect CYR1 binding or the production of cAMP, but did inhibit binding with the SH3 fragment of ABP1p. It was proposed that this reduction in CAP's affinity for SH3 domains was caused by a reduction in CAP dimerisation and it was found that the same mutants were less able to co-precipitate with GFP-tagged CAP (which was larger and therefore easy to differentiate) *in vitro* (Yu *et al* 1999). This suggests that, in yeast, the dimerisation of CAP is required for correct localisation to F-actin and that residues in the N-terminus are essential for this activity. There is however also evidence that the C-terminus of CAP participates in dimerisation as yeast-two hybrid experiments have shown that the amino terminal domain of human CAP1 is able to interact both with itself and with the carboxyl-terminus (Hubberstey *et al* 1996). In addition the carboxyl terminus of yeast CAP may co-immuno-precipitate with both itself and an N-terminal fragment (Zelicof *et al* 1996). The nature of CAP complex formation is thus likely to be complex and involve both ends of the protein. *In vitro* experiments (both gel filtration and chemical cross-linking) have shown that CAP may self associate to form higher order structures (Yang *et al* 1999 and Ksiazek *et al* 2003). The isolation of a native yeast CAP complex has given greater insight into the *in vivo* form of CAP molecules (Balcer *et al* 2003). The complex was found to have a mass of between 587 and 645Kda with CAP and G-actin being present in equal molar ratios. This range would permit the complex to contain six CAP molecules and six bound actin monomers and rotary-shadowing (coupled with electron microscopy) has revealed it to consist of a core region surrounded by lobes of a size equivalent to G-actin. It is therefore proposed that the core is comprised of multimerised CAP proteins with their actin-interacting regions facing outwards. In addition it has been demonstrated that CAP isoforms i.e. human CAP's 1 and 2 may form heterodimers (Hubberstey *et al* 1996), although the functional significance of this association is unclear.

The functional homology and interaction between CAP and Profilin has already been discussed in section 1.5.2 but there is a evidence that the two proteins interact with each other directly as Birch Profilin has been shown to be able to bind a poly-proline domain equivalent to that of CAP *in vitro* (Domke *et al* 1997). It is believed that this interaction occurs through the P1 domain of CAP family members. In addition, the P1 domain of Human CAP1 has also been shown to bind the Abl Tyrosine Kinase via the enzymes SH3 domain. CAP was simultaneously able to bind actin (Freeman *et al* 1996). This is interesting as the disruption of the Abl locus in Drosphila embryos has been shown to result in alterations in F-actin organisation and to genetically interact with CAP in inducing this effect (Baum and Perrimon 2002). The binding and genetic interaction between the two proteins is suggestive of some degree of functional co-operation. A reduction of CAP expression has been shown to result in the mis-localisation of Abl (Baum and Perrimon 2002) and so it is likely that CAP is required for Abl targeting.

The effect of the interaction between CAP and Cofilin family members upon Actin dynamics has already been discussed in section 1.5.2 but the exact biochemical basis of the binding is unclear. It has been proposed that it is dependant upon the N-terminal of CAP as neither Cofilin nor the Cofilin-Actin complex was able to bind the C-terminus of CAP in isolation Moriyama and Yahara 2002), although this may merely demonstrate a requirement for dimerisation. The same study revealed that an expressed fragment of Human CAP1's N-terminal was indeed able to bind the cofilin/actin complex but was not able to interact with either protein in isolation. It appears that the localisation of Cofilin to cortical F-actin is dependant upon its interaction with CAP as it has been demonstrated that Cofilin forms cytoplasmic aggregates with G-actin and is absent from the cell cortex in CAP deficient human cells (Bertling *et al* 2004).

The activity of many proteins is modified via phosphorylation, where a kinase will add a phosphate group to a specific site upon the target protein. The addition of the phosphate may alter the targets ability to bind other proteins or substrates, or in the case of many enzymes affect its catalytic activity. Such modification may be stimulatory or inhibitory and is reversed by the catalytic activity of a phosphotase, which will remove the phosphate group. A sequence analysis of *Arabidopsis* CAP1 that highlights potential phosphorylation sites is presented in Chapter 3, but there is some existing evidence that CAP family

members are regulated by phosphorylation. The effect of reducing CAP expression in developing *Drosphila* embryos is described fully in 1.5.5 but in essence, nurse cells lose their cortical F-actin whereas the adjacent ovaries deposit excessive actin aggregates (Baum *et al* 2000). A similar phenotype was observed when the catalytic-subunit of Protein Kinase A (PKA) was mutagenised and a significantly stronger phenotype was observed in a *cap/pka* double mutant. The cap/pka phenotype is highly distinctive and so it is thought likely that CAP and PKA are both members of the same signalling cascade (Baum *et al* 2000). It is therefore *possible* that CAP is a substrate of PKA and that its regulation is dependant upon PKA-mediated phosphorylation. This is especially interesting when the dependence of PKA activity upon cAMP and the involvement of CAP (in yeast at least) in cAMP production is considered. A hypothetical regulatory negative-feedback mechanism where active CAP co-operates in the accumulation of cAMP until it is phosphorylated by cAMP-activated PKA and is then unable to further stimulate the adenylate cyclase may be considered a realistic possibility. More solid evidence for the phosphorylation of CAP is provided by the interaction demonstrated by the *Letinus edodes* CAP N-terminus and a native 14-3-3 protein (Zhou *et al* 2000). The 14-3-3 proteins represent an important and diverse family of signalling proteins that are believed to associate with phosphorylated binding sites upon target proteins (reviewed by Roberts 2003). Experiments with the fission yeast *S.pombe* have shown the interaction between CAP and 14-3-3 proteins to be conserved although the regulatory and physiological relevance is unclear. This is potentially a significant discovery as the binding of 14-3-3 proteins to targets can mediate a wide variety of effects including stimulation, inhibition, translocation and conformational changes amongst others. The activity of 14-3-3 proteins has been shown to be effective in regulating the activity of many plant proteins, an example being the metabolic enzyme Nitrate reductase is inhibited following 14-3-3 binding (reviewed by Roberts 2003).

1.5.4 CAP in Higher Plants.

The first higher plant CAP family member was identified by screening a *Gossypium hirsutum* (Cotton) fibre cDNA library (Kawai *et al* 1998). Cotton fibres are single cells that can elongate upto 3000 times their diameter (Basra

and Malik 1984) and so provide a good model for identifying genes involved in cytoskeletal organisation. *Gossypium* CAP was sequenced and was found to show 38% homology to Human CAP1 with a greater degree of conservation in the carboxyl-terminal (actin-regulating) domains of the protein. Analysis of *Gossypium* mRNA levels revealed that transcription of CAP was significantly higher in young fibres, which is suggestive of a role in cell elongation (Kawai *et al* 1998).

Analysis of the published higher plant genomes suggests that CAP is highly conserved between species (there is a 76% homology between *C.hirsutum* and *A.thaliana*) and exists as a single copy gene in all plants examined to date (see Section 3.1). The *Arabidopsis thaliana* CAP gene (AtCAP1) was found to be able to complement both the rich media and temperature sensitivity of Cap$^-$ *S.cerevisae* cells, with the carboxyl-terminus in isolation being able to restore the wild-type cell morphology (Barrero *et al* 2002). These results strongly suggest a degree of functional conservation between the yeast and higher-plant CAP's. The same study showed that over expression of AtCAP1 in plants led to a reduction in leaf and petiole size whereas over-expression in cultured Tobacco cells inhibited mitosis and led to a net loss of actin filaments. The reduction in organ size was a result of both reduced cell division and reduced cell size. The over-expression of CAP in *Nicotiana tabacum* yielded comparable results (Barrero *et al* 2003). These observations suggest that the higher plant CAP's play a role in regulating the actin cytoskeleton in a similar way to the CAP's of animals and yeast; a point further supported by the discovery that AtCAP1 was able to bind G-actin via its carboxyl-terminus (Barrero *et al* 2002)

1.5.5 The Role of CAP in Development.

The importance of CAP family members to both cAMP signalling and regulation of the actin cytoskeleton make it likely to be a developmentally important protein with a role in a wide variety of processes. Examination of CAP expression in mammalian systems shows differential expression programs for the two isoforms. CAP1 is highly expressed in brain, kidney, liver and the small intestine of rats, whereas CAP2 was found to be highly transcribed in testes and was entirely absent from the liver and spleen (Swiston *et al* 1995). Further

evidence for regulation of CAP expression is provided by studies of *Xenopus laevis* embryo's where the single CAP isoform (xCAP1) was initially found to be highly up-regulated at the dorsal site of the animal pole at the blastula stage (Khosrow Shahian *et al* 2002). Expression was confined to the mesoderm and precursors of the eye during later stages of development (i.e. gastrulation and neurulation). Studies of the developing Zebrafish embryo have also shown CAP expression to be limited to specific tissues, with early expression being confined to the anterior mesoderm and later expression being especially prominent in muscle-precursor cells (Daggett *et al* 2004). Spatial and temporal regulation of expression are pre-requisites for developmentally significant genes and demonstrate that CAP is involved in more than merely house-keeping.

The concept of cell polarity is important for development, both as a precursor to asymmetric division and as a mechanism for the generation of specialised cell types. The differential transport of mRNA's to the cell poles is believed to be an important in the establishment of polarity and is a microtubule driven process. However, the application of actin depolymerising drugs also disrupts mRNA transport by permitting premature cytoplasmic streaming (Theurkauf 1994). The knockout of CAP in both *Drosophila* and *Saccharomyces* resulted in specific mRNA's (*oskar* and *ASH1* respectively) becoming mis-localised and in the inability of the cell pole to retain the mRNA, i.e. the mRNA appears to diffuse away from the site of delivery (Baum *et al* 2000). The experimenters propose that a lack of CAP resulted in the failure of the cortical actin cytoskeleton to organise correctly; this resulted in both a failure to correctly align actin filaments and microtubules (hence the mis-localisation of mRNA) and the absence of an actin-based structure to tether the mRNA.

The knockout of CAP in *Drosophila* also resulted in cellular actin phenotypes including the deposition of dense F-actin towards the posterior of the egg-chamber and at the cortex of developing eggs (Baum *et al* 2000). The formation of these additional structures within the CAP mutant is accompanied by the simultaneous loss of F-actin structures from other structures, such as the cortical actin network that, in the wild-type, lies under the nurse-cell membrane. This is believed to be a result of differential CAP activity as opposed to the product of a limited actin pool as other mutants that accumulate F-actin within the oocyte (such as the *twinstar* cofilin knockout) do not lose other actin structures throughout the egg-chamber. Antibody staining revealed that CAP

was localised to the oocyte within the wild-type and so it was concluded that CAP serves to inhibit the polymerisation of actin within developing eggs but not elsewhere within the egg chamber.

Further studies of *Drosophila* have concentrated upon the follicular epithelium (the tissue surrounding the developing egg sack) and have shown that the knockout of the CAP gene also results in the accumulation of ectopic F-actin, although this was concentrated at intercellular junctions towards the apical pole of the cell (Baum and Perrimon 2002). The same study revealed that the knockout of actin polymerisation promoting factors including a profiln homolog (Chickadee) and Ena (an Abl Tyrosine Kinase binding protein) in a CAP mutant background prevented the formation of Actin aggregates, which suggests an antagonistic role to that of CAP. The localisation of Ena suggests that it may be specifically responsible for the promotion of actin polymerisation at cell-cell contacts, whereas Profilin has a more generalised distribution, although the correct localisation of Ena was also found to be CAP dependant (Baum and Perrimon 2002). These results suggest that the spatial regulation of actin formation in Drosophila egg sacs is a complex process involving multiple proteins, although in can be assumed that, at the level it is expressed, wild-type CAP acts as an inhibitor of Actin polymerisation. This is in contrast to results achieved in the embryos of Zebrafish (*Danio rerio*), where a reduction in CAP expression significantly reduced the amount of cortical F-actin in Polster cells (Daggett *et al* 2004). The Polster represents a small group of highly motile cells; they regularly extend and retract both filo- and lamellipodia, which suggests that these cells must exhibit a high level of actin turnover. In addition, they have been shown to migrate beyond the animal pole at a defined developmental stage. A reduction of CAP expression reduces the distance achieved by the migration without altering Filopodia/Lamellipodia dynamics (Daggett *et al* 2004).

In summary it can be said that the role in CAP in development is extremely complex and is dependant upon interactions with other actin-modulating proteins. It is also likely that the effect of CAP varies between cells types and at different developmental stages. One general theme however is the conserved importance of CAP to cell extension and the maintenance of polarity. It has been proposed that these, via organisation of the actin cytoskeleton, represent universal functions for CAP family members (Hubberstey and Mottillo 2002).

The discovery that over-expression of *SNC1*, a synaptobrevin homolog, from *S.cerevisiae* could partially complement the *cap* phenotype (wild-type cell size was restored) was the first suggestion that CAP may play a role in vesicle-trafficking (Gerst *et al* 1992). Characteristics associated with the N-terminal functions of CAP, such as responsiveness to Ras were not complemented by the over-expression of *SNC1* and its deletion had no visible phenotype, suggesting that its function is not essential. A mutation in CAP that inhibits the endocytosis of pheromones provides further evidence for an additional CAP function (Wesp *et al* 1997). This study also revealed that the deleterious effects of CAP-deficiency upon vesicle trafficking were confined to endocytosis as the intracellular transport of products from the Endoplasmic Reticulum, to the Golgi Body and onto vesicles was not inhibited. It is believed that CAP participates in endocytosis via a protein complex as the complete elimination of CAP expression does not affect the uptake of pheromones (Wesp *et al* 1997), therefore it has been proposed that the CAP mutation mediates its effect by disrupting an endocytosis complex, i.e. it acts as a dominant negative mutant. In addition CAP has been shown to interact with ABP1p (which has been shown to interact with dynamin; a provider of motile force) and Rvs167p, which is a yeast homolog of amphiphysin and is believed to play a key role in regulating endocytosis via dynamin (Drees *et al* 2001, Hubberstey and Mottilo 2002). In addition there is evidence that CAP may also play a role in promoting endocytosis within higher Eukaryotes as the RNAi induced removal of CAP from cultured human cells reduced their ability to correctly endocytose rhodamine-conjugated Transferrin by approximately 45% in comparison to wild-type cells (Bertling *et al* 2004).

1.6 Aims and Objectives of this Study.

It is hoped that the previous sections of this introduction have successfully communicated the importance of the Actin cytoskeleton to the development and function of Eukaryotic organisms and the particular relevance of the cytoskeleton to higher plant development and function. The Cyclase Associated Protein (CAP) family represents an important group of actin regulating proteins ; the activity and function of which (especially in higher

plants) is poorly understood. The interaction of yeast CAP's with adenylate cyclases (Field *et al* 1990) and their role in facilitating Ras-induced cAMP production is interesting given the paucity of information regarding cAMP signalling in plant cells. Higher plants would be unique amongst all kingdoms were they not to utilise cAMP signalling and at least some plant tissues have been shown to respond to the addition of exogenous cAMP (Moutinho et al 2001). Therefore CAP, given the importance of cAMP to the signalling mechanisms in other organisms and it's diverse range of down-stream targets, is an extremely important protein. The CAP family occupies a unique niche as they are ideally positioned to mediate cross-talk between the cAMP pathway (an important signalling mechanism) and the actin cytoskeleton (a critical effector mechanism that must be coupled to signalling pathways by interacting proteins).

This study had multiple aims, all of which were intended to increase understanding of actin-regulation within the plant cell and if possible to relate regulation to cyclic AMP signalling. It was intended to initially analyse the *Arabidopsis* CAP sequence *in silico* in an attempt to garner details about its function by comparing it to better characterised CAP genes and by studying its distribution throughout the plant. Secondly it was intended to examine the role played by CAP in whole-organism development by studying the effect of eliminating CAP expression upon plant morphology. In particular the effect of CAP knockout upon actin formation and distribution would be studied in a variety of cell types. Thirdly the CAP gene was to be cloned and expressed, so allowing it's *in vitro* interaction with actin to be studied and compared to the other CAP family members. The Arabidopsis CAP gene was also to be used in a series of Yeast-Two-Hybrid experiments in an attempt to study interactions with other known actin-binding proteins. Finally CAP was to be used as a tool to identify potential adenylate cyclases within the higher plant system and to study the effects of disrupting cAMP signalling upon plant development.

Chapter II: Materials and Methods

2.1 Materials

2.1.1 Chemicals

Unless otherwise stated all chemicals used were of an analytical grade and were supplied by the Sigma Chemical Company (Gillingham, England) or BDH (Poole, England).

2.1.2 *E.coli* Strains.

DB3.1 F^- *gyr*A462 *end*A1 D(sr-recA) *mcr*B *mrr hsd*S20(rB^-, mB^-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*-5l^- *leu mtl*-1

DH5α *supE*44 σ*lac*U169 (∅80*lacZ*σM15) *hsdR*17 *RecA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1

BI21(DE3) F^- *ompT* $hsdS_B(r_B^-m_B^-)$ *gal dcm* (DE3)

XL1-BLUE –*rec A1 end A1 gyrA96 thi-1 hsdR17 suoE44 relA1 lac*[F' *proAB* $lacI^qZ\Delta M15$ Tn *10* (Tet^r)

2.1.3 *Agrobacterium tumefaciens* Strain

C58C3 An industrial strain with a C58 background. Resistant to Nalidixic acid and Streptomycin.

2.1.4 *Saccharomyces cerevisiae* Strains

AH109 *MATa trp*1-901 *leu*2-3 112 *ura*3-52 *his* 3-200 *gal* 4Δ *gal*80Δ LYS2 $GAL1_{UAS}$ – $GAL1_{TATA}$ –HIS3 $GAL2_{UAS}$-$GAL2_{TATA}$ –ADE2

Y187 *MATα ura*3-52 *his*3-200 *ade*2-101 *trp*1-901 *leu*2-3 112 gal4Δ *met- gal*80Δ URA3 $GAL1_{UAS}$ –$GAL1_{TATA}$ –*lacZ*

2.1.5 *Arabidopsis thaliana* Lines

LINE NAME	SOURCE	BACKGROUND	DESCRIPTION
Wild Type	Lehle Seeds	Columbia	
SALK 112802	Salk Institute	Columbia	CAP tDNA Insert
GABI-KAT 453G08	Max Plank Institute	Columbia	CAP tDNA Insert
GFP-FABD2	Bonn	Columbia	For *in vivo* actin imaging
635 A5	Syngenta	Columbia	CAP tDNA Insert

2.2 Methods

2.2.1 CLONING TECHNIQUES

2.2.1.1 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to generate DNA fragments suitable for ligating or recombining into a plasmid vector. A Hybaid (Ashford, UK) Omni-E thermal cycler, with a heated lid, was used for these purposes. Typically a reaction would contain the Forward and Reverse primers (at a concentration of 0.5μM), 1.5μM $MgCl_2$, 200μM dNTP's, approximately 1ng of template DNA, 2 Units of a suitable polymerase and a buffer appropriate for the enzyme in use. Typically *pfu* (Bioline London, England) would be used for targets less than 2Kb in length, whereas Expand (Roche Lewes, England) was used for longer targets due to increased fidelity. Reactions were made up to a volume of 50μL using sterile distilled water.

Reactions consisted of an initial 60 second 94°C denaturation followed by 35 repeated cycles of denaturation (94°C) for one minute, primer annealing for one minute and an extension stage. The annealing temperature was dependant upon the primers in use and varied between 59 and 62°C. The extension time was dependant upon the length of the target and was calculated on the basis of 1Kb per minute (at 68°C) with Expand and two minutes (at 72°C) with *Pfu*.

2.2.1.2 Agarose Gel Analysis

Agarose gels were used for the visualisation and isolation of PCR products and restriction digested fragments. Generally 1% Agarose gels were made by melting a suitable amount of electrophoresis grade Agarose in modified TAE buffer (40mM Tris acetate, 1mM EDTA, pH 8.0) in a microwave. The molten agarose was allowed to cool before Ethidium bromide to a final concentration of 0.5µg ml^{-1} was added. Finally the gel was poured into a clean gel tray with a suitable comb in place and allowed to set.

An appropriate amount of 10x loading buffer was added to each DNA sample prior to addition to the well. Gels were run in modified TAE buffer at 100v and progress was monitored by observing the loading dye's migration. Finally the gels were visualised under UV illumination with a BioRad Gel Dock 2000 (BioRad, Hemel Hempstead, England) system and were photographed.

2.2.1.3 Gateway Cloning and Sub-cloning Reactions

The Invitrogen Gateway Cloning System was used for much of the cloning described in this thesis as it permits the rapid sub-cloning of fragments into a variety of destination vectors whilst maintaining both frame and orientation. In essence the first stage of the system (the BP reaction) utilises the phage λsite specific recombination mechanism to inset an *attB* flanked PCR product into an *attP* site within a donor vector to produce and entry clone. Att sites were added to

the PCR products by incorporating them into the primers. An overview of the Gateway system is given in Fig2.1.

Negative selection is provided by the *ccdB* gene, which lies between the donor vector's *attP* sites and encodes the CcdB inhibitor of DNA Gyrase. A successful recombination reaction will result in the replacement of this toxic-cassette with the *attB* flanked PCR product and so only the recombined plasmid will permit *E.coli* reproduction.

A typical BP reaction will consist of 4µL of 5x BP reaction buffer (Invitrogen, Paisley, Scotland), 4µL of BP Clonase enzyme mix, 300ng of Donor vector (usually 2µL) and an equimolar quantity of *attB* flanked PCR product. The reaction volume was made up to 20µL by the addition of TE Buffer (pH 8) and was incubated at 25°C for 1 hour. Termination of the reaction was performed by the addition of 4µL of Proteinase K (Invitrogen, Paisley, Scotland) and subsequent incubation at 37°C for 10min. Successfully recombined plasmids were propagated via transformation into *E.coli* (DH5α) and selection on an appropriate antibiotic.

The fragment of interest could then be transferred to a destination plasmid, such as an expression vector or a yeast-two-hybrid vector via a LR recombination reaction. This transfers the gene lying between the *attL* sites of the entry clone into the *attR* sites of the destination vector with new *attB* sites being generated on either side of the gene. The *ccdB* toxic gene will again be excised from the destination vector and so provides negative selection. A typical LR reaction would consist of 4µL of LR 5x reaction buffer, 4µL of LR recombinase enzyme mixture, 300ng of destination vector, an equimolar amount of entry clone and would be made up to 20µL with TE Buffer (pH 8). Again the reaction would be incubated at 37°C for one hour and would be terminated by the addition of Proteinase K.

2.2.1.4 Addition of 3' Adenine Overhangs to PCR Products

PCR products generated by Taq polymerases that lack the ability to add template independent 3' adenine residues (such as *Pfu*) require the addition of these bases before they could be used in TA overhang-dependant cloning reactions. Initially the PCR product was purified using a QIaQuick PCR clean-up kit (Qiagen, Crawley, England) and eluted in a volume of 30µL of elution buffer. This was then added to

Figure 2.1: The Gateway Cloning System

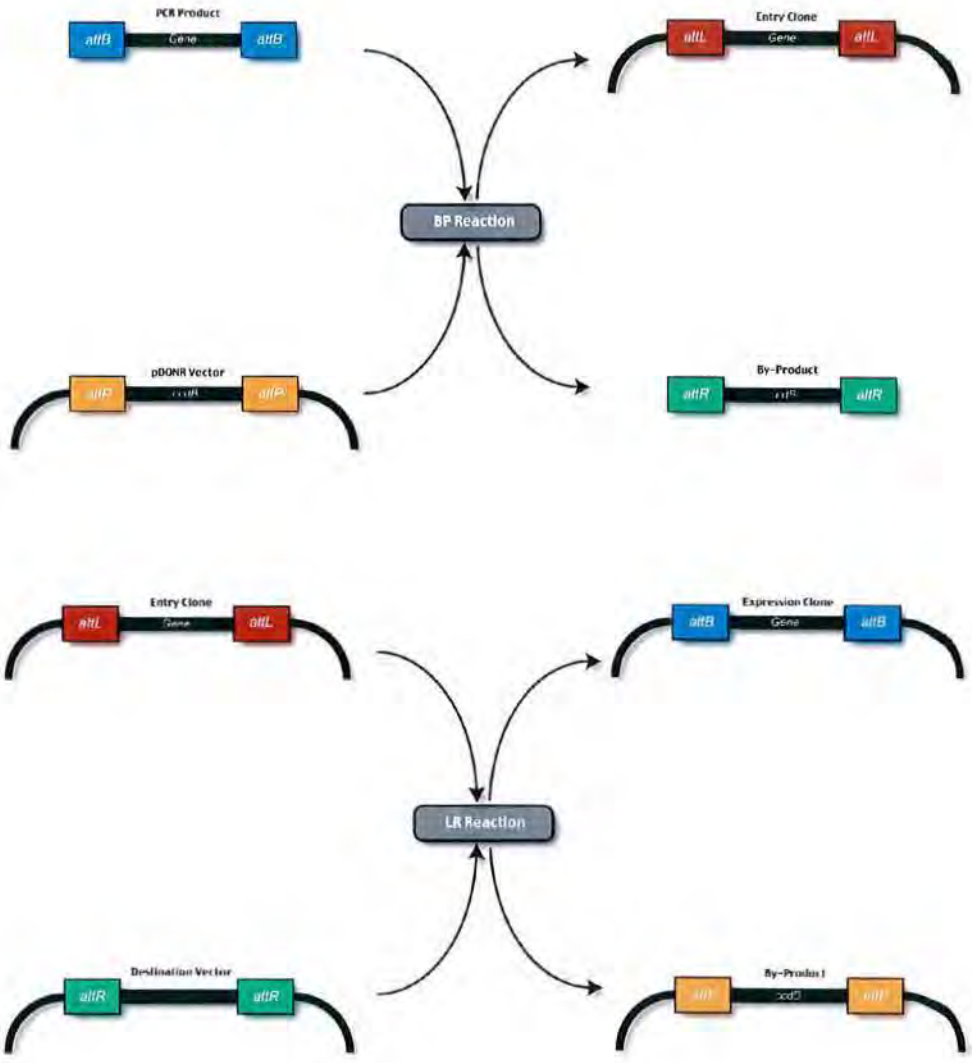


Fig 2.1: A product would usually be amplified by PCR with primers containing the attB sites and then cloned into an entry vector by a BP reaction. The introduction of the PCR product into the entry vecor results in the displacement of the ccdB cytotoxicity gene. The entry vector may then be used to transfer the PCR product to a useful destination vector (such as an expression plasmid) via a LR reaction.

a reaction containing 5µL of 10x Taq PCR Buffer (Promega), 5mM Magnesium Chloride, 1mM dATP, 5 units of *Taq* (Promega). The volume was made up to 50µL by the addition of SDW and incubated at 70°C for 30 min. The enzyme was removed by the subsequent use of a QIAquik PCR clean-up kit (Qiagen).

2.2.1.5 Ligation Cloning and Sub-cloning

The initial cloning of non-Gateway PCR products was achieved by ligating the fragment into the pGEMT-Easy vector (Promega, Southampton, England) via an AA-TT interaction at both termini. The reactions were performed in a volume of 20µL and contained 10µL of 2x Rapid ligation buffer (Promega), 3U of T4 Ligase (Promega) in addition to the PCR fragment and pre-linearised vector in a 3:1 molar ratio. The reaction was incubated for 2 hours at 20°C. The post-ligation mix was used to transform competent *E.coli* and the amplified plasmid was analysed by digestion and sequencing.

DNA fragments could also be sub-cloned into further vectors via ligation. Linerisation of the vector at an appropriate site was achieved via a restriction digest (see 2.2.1.7). Vector self-ligation was prevented by de-phosphorylation of the vector. The 5'-end of the cut vector was de-phosphorylated by the use of Calf Intestinal Alkaline Phosphatase (CIAP- Promega). This reaction required 10µL of 10x CIAP reaction buffer (Promega), 2µg of digested vector DNA, 1 unit of CIAP and were made up to a volume of 100µL with SDW. It was incubated at 37°C for 30min before an additional unit of CIAP was added and was then incubated for a further 30min. The addition of 2µL of 0.5M EDTA and subsequent incubation at 65°C for 20min terminated enzymatic activity. A QIAquik PCR clean up kit (Qiagen) was used to recover the plasmid DNA, which could then be used in a standard ligation reaction as previously required.

2.2.1.5.1 Preparation of Chemo-competent E.coli

Cells from a frozen *E.coli* glycerol stock were streaked onto a LB plate and incubated at 37°C for approximately 15hrs. Single colonies from the plate were

used to inoculate 3ml of Psi broth, this was also incubated for approximately 15hrs at 37°C but was shaken at 200rpm to ensure aeration. 1.5ml of this culture was then used to inoculate 150ml of Psi broth that was grown at 37°C with shaking until an optical density (OD) at 550nm of 0.5 was achieved.

The culture was incubated on ice for 15min before being pelleted by centrifugation at 1000g for 10 min. The pellet was gently re-suspended in 60ml of Buffer TfbI (30mM Potassium acetate, 100mM Rubidium chloride, 10mM Calcium chloride, 50mM Magnesium chloride, 15% Glycerol, pH 5.8). The cells were incubated on ice for a further 15min and were subsequently pelleted as before. The pellet was gently re-suspended in 6ml of Buffer TfbII (10mM MOPS, 75mM Calcium chloride, 10mM Rubidium chloride, 15% Glycerol, pH 6.5) before being incubated on ice for another 15min. The cells were then aliquoted into pre-chilled micro-centrifuge tubes, snap frozen in liquid nitrogen and stored at –80°C.

2.2.1.5.2 Transformation of Chemo-competent E.coli

50µL of Chemo-competent cells were thawed on ice prior to the addition of 5µL of a ligation or recombination reaction. The cell/DNA mixture was incubated for a further 30min on ice before being heat-shocked for 40s at 42°C. The cells were then transferred to ice for a further 2min before 500ml of LB broth was added and the reaction tube was transferred to 37°C for 1 hour. This permits recovery and allows expression of any antibiotic resistance genes encoded by the plasmid to begin.

Centrifugation (1000g for 10min) was then used to pellet the cells prior to re-suspension in 50µL of LB. The cells were then transferred to the surface of an LB plate (containing 1% Agar) and were dispersed across the surface with a sterile spreader. Each 25ml plate would contain an antibiotic appropriate to the plasmid being selected for and may also have contained 1mM IPTG (Melford, Ipswich, England) and 80µM X-gal (Melford) so as to permit blue-white selection of transformants.

2.2.1.6 Plasmid Isolation

Individual colonies were selected from a transformation plate and were used to inoculate 3ml of liquid LB media (containing an appropriate antibiotic). These cultures were incubated for approximately 15hrs at 37°C with constant shaking at 200rpm. The cells were pelleted via centrifugation at 1000g for 10min before the plasmid was recovered by the use of a Miniprep kit. A GenElute Plasmid Miniprep kit (Sigma, Poole, England) was used for *E.coli* extractions whereas a Wizard SV Plus kit (Promega) was used on *Agrobacterium* samples. In both cases the manufacturers directions were followed and in both cases the plasmid was eluted in 30µL of the manufacturers elution buffer.

2.2.1.7 Restriction Digests

A typical restriction digest would contain 1µg of DNA, 2 units of each restriction endo-nuclease, 2µL of the manufacturers recommended 10x digest buffer and would be made up to a volume of 20µL with SDW. Reactions would be incubated at 37°C for 1 hour and would be terminated by 10min incubation at 70°C. The results were analysed on an Agarose gel.

2.2.1.8 Automated DNA Sequencing

Sequencing reactions were performed by the staff of DBS Genomics (Durham); approximately 300ng of template DNA was required for each reaction. Sequence specific primers were supplied at a concentration of 3.2pM.

2.2.2 ISOLATION AND ANALYSIS OF PLANT GENOMIC DNA

2.2.2.1 The Propagation of Arabidopsis thaliana

Arabidopsis seeds were sterilised by a 2 minute wash in 70% Ethanol followed by 2 minutes in a 5% Hypochlorite/ 0.2% Tween solution. The seeds were

Figure 2.2: The Genotyping of T-DNA Insertion Lines

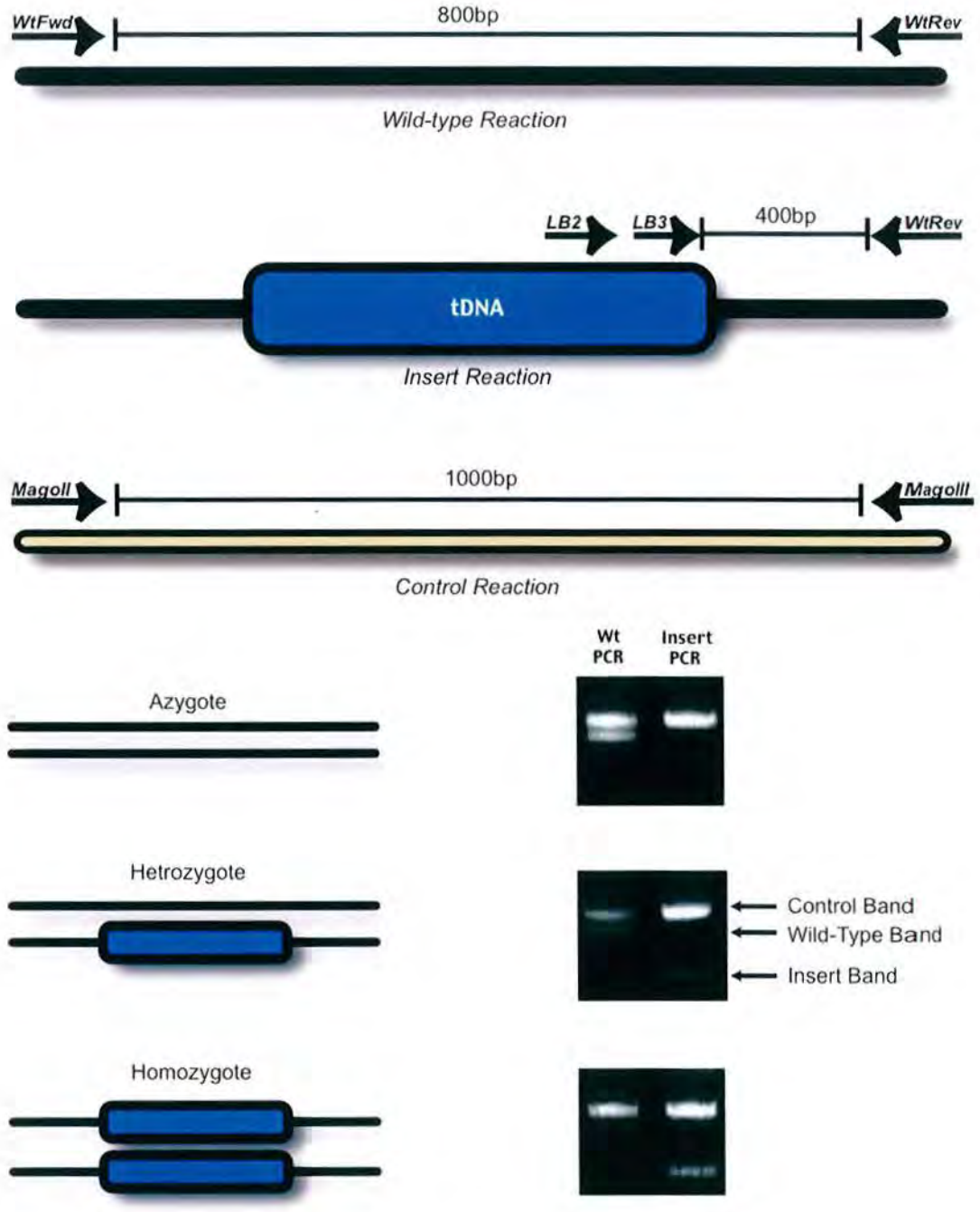


Fig 2.2: An overview of the Genotyping of tDNA Insert Lines. The Wild-type reaction utilises gene-specific primers and confirms the presence of at least one wild-type allele. The Insert reaction requires one of two tDNA specific primers (LB2 or LB3) and one gene specifc primer It confirms the presence of at least one tDNA-disrupted allele. A pair of control primers were present in each reaction to prevent false negatives.

then washed 10 times with 1ml of SDW. This sterilisation was conducted in a flow hood. The seeds were placed on a MS Agar (0.7% Agar with Murashige and Skoog salts, Gibco, Paisley, Scotland) and were vernalised for 4 days at 4°C. The seeds were then germinated under constant illumination with the plates being vertically orientated so as to allow root development to be observed. They were subsequently transferred to individual pots containing a 75% Compost/25% Silver Sand mix and grown under a 16hr light regime at 18°C. Initially plants were treated with Intercept 70WG (Scotts, Marysville, Ohio, USA) at 0.2g L^{-1} in tap water, in an attempt to prevent fly infestation.

2.2.2.2 Genomic DNA Extraction

The method of Edwards was used (Edwards *et al* 1991). Leaves were collected with particular care being taken to avoid cross-contamination and were immediately flash frozen in liquid N_2. The samples were then homogenised using a small grinder and further liquid N_2 before being thoroughly re-suspended in 400µL of Extraction Buffer (200mM Tris, 250mM Sodium chloride, 25mM EDTA, 0.5% SDS, pH 7.5). Debris was removed by centrifugation at 16,000g for 20min, with 300µL of the supernatant then being carefully removed and added to 300µL of pre-chilled Isopropanol. The tubes were incubated to ice for a further 5 min before the precipitated DNA was pelleted by 10 min centrifugation at 16,000g. The pellet was washed with 200µL of 70% Ethanol, to remove salts, and was then air-dried. The DNA was re-suspended in 30µL of TE Buffer (pH8) and stored at –20°C.

2.2.2.3 Genotyping of tDNA Insert Lines.

Genotyping of tDNA Insert plants required two separate PCR reactions, with the first 'wild-type' reaction being designed to amplify a fragment from the gene of interest straddling the insertion site. Therefore a product of the correct size will only be produced if there is at least one wild-type copy of the gene present. A second 'Insert' PCR used a primer specific to the left border of the tDNA and a wild-type primer in the correct orientation. Therefore a product would only be amplified in the

tDNA was present. In both a cases a pair of control primers that amplified and unrelated DNA fragment were included in each tube in an attempt to prevent false negatives. An overview of this procedure is given in Figure 2.2 and the primers used are detailed in Appendix 1.

2.2.3 Protein Expression Methods

2.2.3.1 Induction of Expression

Expression constructs were transformed into a suitable *E.coli* strain (such as BL21-DE3) and the resultant colonies were used to inoculate 3ml overnight LB cultures. These cultures would then be used to inoculate a further litre of LB media with an appropriate antibiotic also being present; so as to select for the expression clone. The cultures were grown at 37°C with shaking (200rpm) until an OD_{600nm} of 0.6 was achieved. At this point IPTG (Melford) (to a final concentration of 1mM), was added to the culture in order to induce expression of the protein encoded by the plasmid (expression was under the regulation of the *lac* operon). The period of induction, the *E.coli* strain used and the incubation temperature were all optimised through experimentation. Details of the expression of AtCAP1 are given in Appendix III.

2.2.3.2 Isolation of Protein

Expression cells were pelleted by centrifugation for 10 minutes at 9000g and were then re-suspended thoroughly in 10ml of pre-chilled binding buffer (10mM Imidazole, 10mM diSodium hydrogen orthophosphate, 10mM Sodium dihydrogen orthophosphate and 0.5M Sodium chloride, pH7.4). Cells were lysed by sonication for 30secs and the debris was removed by 30min of centrifugation at 48,000g. Proteolysis was reduced by chilling the lysate and by the addition of protease inhibitors: (Pepstatin-1µL per ml, Aprotinin 6.7µL per ml, TAME 5µL per ml, Leupeptin 1µL per ml, PMSF 2.5µL per ml).

The supernatent was loaded onto an equilibrated 1ml HisTrap column (Amersham, Chalfont-St.Giles, England) via a syringe. It was intended that the

desired protein would bind to the column's Nickel ion matrix via a charge-charge interaction between its Histidine tag and the matrix with most other un-tagged proteins not being retained. The column was then washed by running through 10ml each of 40mM, 60mM and 100mM Imidazole solutions in an attempt to remove any weakly interacting proteins. Finally the protein was eluted in 5ml of a 0.5M Imidazole, 0.5M Sodium chloride, 10mM diSodium hydrogen orthophosphate, and 10mM Sodium dihydrogen orthophosphate buffer as Imidazole at this concentration is sufficient to disrupt Nickel-Histidine interactions. The protein would then be dialysed into a suitable buffer (e.g. G-Buffer or PBS) for approximately 6 hours at 4°C.

2.2.3.3 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The integrity and purity of an expressed protein was assessed by running it on a Poly-acrylamide gel using the method of Laemmli (1970). In brief a 10ml 12% resolving gel solution would contain 4ml of SDW, 2.5ml of 1.5M Tris (pH8.8), 4ml of a Protogel 33% Acrylamide solution (National Diagnostics, Hessle, England), 100µL of 10% SDS, 100µL of 10% Ammonium persulphate and 4µL of Tetramethyl-ethylenediamine (TEMED). The solution was poured between two gel plates and allowed to set before the resolving gel and gel-comb (which provides loading wells) were added. The resolving gel was made up to a volume of 4ml per gel and comprised of 2.7ml of SDW, 670µL of Protogel, 500µL of 1M Tris (pH 6.8) 40µL of 10% SDS, 40µL of 10% Ammonium persulphate and 4µL of TEMED.

The gel was submerged in PAGE running buffer (25mM Tris, 250mM Glycine and 0.1% SDS) and up to 15µL of protein sample was loaded in each well. The samples had previously been mixed with 2x Loading buffer (50mM Tris, 100mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10% Glycerol, pH 6.8) and incubated at 100°C for 5 min. This ensured that the samples were fully denatured and reduced. The gel was run at 200v until the dye-front reached the bottom edge of the gel, at which point the resolving gel was excised and was submerged in Coomassie stain (50% Methanol, 10% Acetic acid and 1.25% Coomassie Brilliant Blue) for 30 minutes. The gel was then placed in de-stain solution (10% Methanol

and 10% Acetic acid) until protein bands were clearly visible and finally was scanned

2.2.3.4 Determination of Protein Concentration

The concentration of proteins was determined by measuring the absorbance at 280nm of the protein solution with a UV Spectrophotometer. The instrument had been blanked against whichever buffer the protein was suspended in and a high quality Quartz cuvette was used. The molar concentration of the protein is given by:

$$[\text{Protein}] = \frac{OD_{280}}{\varepsilon}$$

where ε is the protein's extinction co-efficient and is given by:

$$\varepsilon = 1500Y + 5380W$$

Y represents the number of Tyrosine residues and W represents the number of Tryptophan residues in the protein.

2.2.4 BIOCHEMICAL METHODS

2.2.4.1 Generation of Antibodies

Polyclonal antibodies were generated by the injection of expressed proteins into mice (*Mus musculus*) and subsequent collection of the anti-sera. Proteins to be injected were dialysed into Phosphate Buffered Saline (PBS) and diluted to a concentration of 1µg ml^{-1}. The samples were then mixed 1:1 with Freunds Incomplete Adjuvant and thoroughly mixed by vortexing for 5 min.

The injections were performed by the staff of the Life Science Support Unit (Durham) and resulted in each mouse receiving 100µg of protein and therefore 200µL of the PBS/Adjuvant solution. Three BALB mice were used and each mouse

received a course of three injections with two weeks between injections. A sample was taken from the tail of each mouse and assessed (via a Western Blot) for affinity to and specificity for the target protein. Satisfactory performance resulted in the mouse being sacrificed with the serum being isolated, flash frozen in Liquid nitrogen, aliquot and stored and –80°C. Sodium azide was added to a concentration of 0.1% to preserve the antibody.

2.2.4.2 Western Blotting

Proteins were separated by SDS-PAGE (see 2.2.3.3) and transferred onto a Nitrocellulose membrane (Amersham) by electroblotting. The blot was suspended in Transfer buffer (25mM Tris, 200mM Glycine and 30% Methanol) within a vertical blotting tank and a 20V field was applied for approximately 15 hours. The membrane was then washed in SDW and stained with a 0.1% Amido black solution for 10 minutes in order to confirm that protein transfer had occurred. The stain was removed by washing the membrane for 30 min in 2x Tris Buffered Saline with Tween (TBS-T 20mM Tris, 280mM Sodium Chloride, 0.1% Tween, pH 7.4) with continuous changes of buffer.

Non-specific labelling of the membrane was prevented by blocking it with a 5% Fat free Milk (in 2x TBS-T) solution for 30 min. The milk was removed by rinsing in 2x TBS-T for 30 min with three changes of buffer. The primary antibody anti-body was diluted to the correct concentration, which had been previously determined by test blots, with 5% Fat-free milk in 2x TBST-T and applied to the membrane. Usually 1ml of Antibody solution was used and the blot was incubated for one hour at 20°C.

The membrane was again washed with 2x TBS-T for 30min with three changes of buffer before the secondary antibody was added. This was a goat anti-mouse Horseradish peroxidase-conjugated antibody (supplied by Amersham) was diluted 1:5000 in 5% Fat-free milk/2x TBS-T. Again the blot was incubated with 1ml of the antibody for an hour prior to being washed with 2x TBS-T for one hour with six changes of buffer.

One ml of ECL Detection reagent (Amersham) was added to the surface of the membrane and allowed to stand for one minute before the excess fluid was

drained. The membrane was then placed into a Chemiluminescent Gel Doc (Fujifilm, London England), which detected the luminescent product of the peroxidase and therefore the location of bound secondary antibody.

2.2.4.3 Actin Co-sedimentation Assays

Initially the protein of interest was spun at 200,000g in a Beckman 120.1 rotor for 15 min at 4°C to remove any aggregated protein. The concentration of protein remaining in the supernatant was then measured. Polymerisations were performed in a volume of 100µL with both actin (Cytoskeleton Inc, Denver, CO, USA) and protein of interested present at a concentration of 5µM; the volume was made up to 90µL by the addition of fresh G-Buffer (2mM Tris, 200µM ATP, 500µM DTT, 200µM Calcium chloride and 0.002% Sodium azide, pH 8.0). Finally 10µL of a 10x KME polymerisation buffer (100mM Imidazole, 500mM Potassium chloride, 10mM Magnesium chloride and 10mM EGTA, pH 6.5) was added and the reaction was incubated for one hour at room temperature.

The reaction tubes were then centrifuged at 150,000g for 15 min at 15oC to separate polymerised F-actin (and any interacting proteins) from the soluble fraction remaining in the supernatant. The supernatant was removed and mixed 1:1 with protein loading buffer and the pellet was re-suspended in 100µL of G-Buffer and then added to loading buffer. Both samples were then boiled at 100°C for 5 min and analysed on a SDS-PAGE gel (see 2.2.4.2).

2.2.4.4 Transmission Electron Microscopy (TEM)

F-actin filaments were generated by the incubation of 5µM G-actin (Cytoskeleton Inc) in 100µL of G-Buffer+10x KME (as described in 2.2.4.3) for one hour at 20°C. The filaments were then diluted 50-fold in G-Buffer before 20µL was transferred to a carbon-coated Nickel/Copper Electron microscopy grid (Agar Scientific, Stanstead, England). The grid was left for 3 minutes to allow the filaments to settle before excess liquid was removed with blotting paper. A 1% Uranyl acetate solution was used to stain the grid; it was found that staining for 30 yielded the optimum contrast for these experiments. Finally the grids were taken to

the Electron Microscopy Unit (Durham) and were visualised at a range of magnifications.

2.2.5 *In Vivo* Cell Studies

2.2.5.1 Germination of Pollen

Fresh Daffodil (*Narcissus*) pollen was harvested and germinated in a media containing 7.5% Glucose, 1mM Calcium Nitrate, 1mM Boric Acid and 15mM MES (pH 6.7) for 2 hours. Oxygenation of the germinating pollen was ensured by transferring the pollen and media to a Petri Dish; so maximising the surface area. Drugs such as Cytochalasin D (30µM) and Latrunculin B (30nM) were added to the germination media 30 min prior to fixation if actin disruption was required. Germination of the pollen was monitored by examining the pollen tubes under Differential Interference Contrast (DIC) optics.

2.2.5.2 Fixation and Digestion of Pollen Tube Cell Walls

Fixation of germinated pollen tubes was performed at 20°C for 40 min with 5ml of a fixative containing 3.7% Paraformaldehyde, 50mM PIPES (pH 6.8), 2mM Magnesium chloride and 2% Glycerol. The tubes were washed twice for 10 min with Phosphate Buffered Saline (PBS) post fixation. The external cell wall was rendered permeable by digestion with an enzyme mixture containing 1% Cellulase, 1% Maceroenzyme, 1% Pectolyase, 1% Dricelase, 0.4M Mannitol and 5mM EGTA. Protein epitopes were conserved by adding the protease inhibitors PMSF (100mM), Leupeptin (25mg ml^{-1}) and TLSK (37 mg ml^{-1}) to the digestive mixture.

2.2.5.3. Staining and Visualisation of Pollen Tubes.

Pollen tubes were attached to poly-lysine coated cover slips by transferring approximately 50µL of fixed and digested tubes to the surface of the slip and

allowing to dry and settle. The cover slips were incubated with the primary antibody, which had been diluted to the appropriate concentration in PBS+ 0.1% Bovine Serum Albumen (BSA), for one hour.

They were then washed three times in PBS with each wash lasting for 10 min before the secondary antibody was added. This would usually be a TRITC-conjugated anti-mouse monoclonal antibody (Molecular Probes, Paisley, Scotland). The antibody was diluted 1:100 as per the manufacturers instructions and the incubation lasted for one hour. Again the cover slips were washed three times on PBS for 10 minutes each time.

The visualisation of Actin within the pollen tube was achieved by incubating the fixed and digested tubes in Bodipy-conjugated Phallocidin (Molecular Probes). The stain was diluted 1:10 in PBS+0.1% BSA as per the manufacturers directions and was left in place for 30 minutes. Often this step was performed after a primary and secondary antibody staining, so allowing co-localisation between actin and a protein of interest to be studied.

Finally the cover-slips were mounted in VectaShield (Vector Laboratories) and viewed on either a Micro Radiance 2000 (BioRad, Hemel Hempstead, England) or a LSM 510 Meta (Carl Zeiss, Oberkochen, Germany) Laser Scanning Confocal Microscope.

2.2.5.4 The Imaging of GFP-Constructs

Tissues expressing GFP labelled bio-probes were sectioned using a sharp razor blade and mounted upon a microscope slide in distilled water. The samples were imaged using either a Micro Radiance 2000 (BioRad, Hemel Hempstead, England) or a LSM 510 Meta (Carl Zeiss, Oberkochen, Germany) Laser Scanning Confocal Microscope.

2.2.6 The Yeast-Two-Hybrid System

2.2.6.1 The Transformation of Yeast.

The strains AH109 (mat-a) and Y187(mat-α) were resurrected from frozen glycerol stocks by streaking them out onto fresh YPDA plates (20g L^{-1} Tryptone, 10g L^{-1} Yeast Extract, 30mg L^{-1} Adenine hemisulphate, 2% Glucose and 20g L^{-1} Agar, pH 5.8) and incubating for 48hrs at 30°C.

Single colonies were taken from these plates and were used to inoculate 10ml liquid YPDA cultures. These were grown at 30°C with shaking (at approximately 200rpm) in a 250ml flask until an OD_{600} of approximately 0.3 was achieved. This would usually take at least 15 hours. The yeast were then pelleted by centrifugation at 1000g for 10 minutes and re-suspended 100ml of SDW. They were subsequently pelleted again before being re-suspended in 1.5ml of 0.4M Lithium acetate (in TE Buffer) and pelleted again.

This was followed by re-suspension in 0.5ml of Lithium acetate and 100µL of this Yeast suspension was added to 160µg of denatured Salmon sperm carrier DNA, 1µg of plasmid DNA, 10µL DMSO and 600µL of 0.4M Lithium acetate (dissolved in a 1:1 PEG 4000/TE Buffer solution). The transformations were then incubated at 30°C for 30 minutes before being transferred to 42°C for a further 30 minutes. Finally the yeast was pelleted via centrifugation and re-suspended in 1ml of SDW before 100µL was spread onto a selective SD plate.

The plasmid vectors confer the ability to bio-synthesise an otherwise essential nutrient and therefore allow growth upon media lacking that nutrient. The Binding Domain (BD) vector (pAS2-1GW) includes the *LEU2* gene that is responsible for the synthesis of Leucine, whereas the Activating Domain (AD) vector (pAct2-1GW) permits the synthesis of Tryptophan by including the *TRP1* gene. An overview of the Yeast-Two-Hybrid system is given in Figure 2.3.

2.2.6.2 Auto-activation Assays.

The autonomous activation of the reporter genes via a direct interaction between the BD construct and the reporter gene's promoter is a major source of false positives in the Yeast-Two-Hybrid system. This problem is avoided by assaying yeast containing solely the BD construct for reporter gene activity. For the purposes of this study the BD constructs were transformed into the strain AH109,

Figure 2.3: The Principles of Yeast Two-Hybrid.
(Based upon a figure from the Clontech MATCHMAKER GAL4 Two-Hybrid System 3 Users Manual)

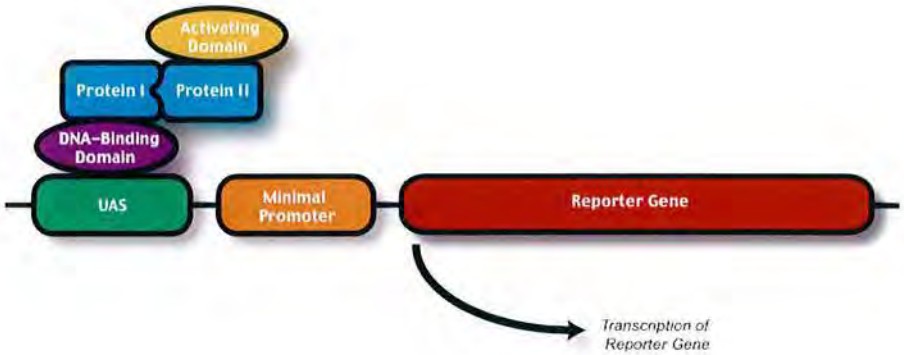


Fig 2.3a: The interaction between the two proteins of interest will result in the assembly of the GAL4 Transcription factor and activation of the reporter gene.

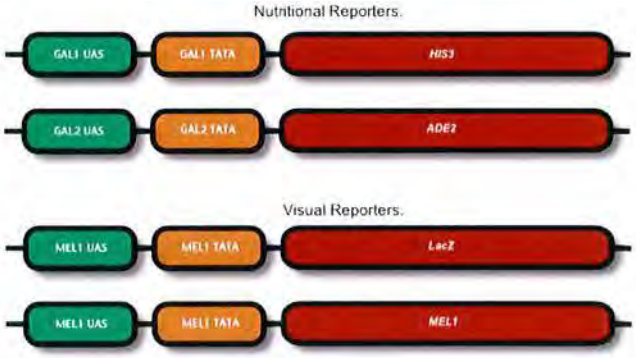
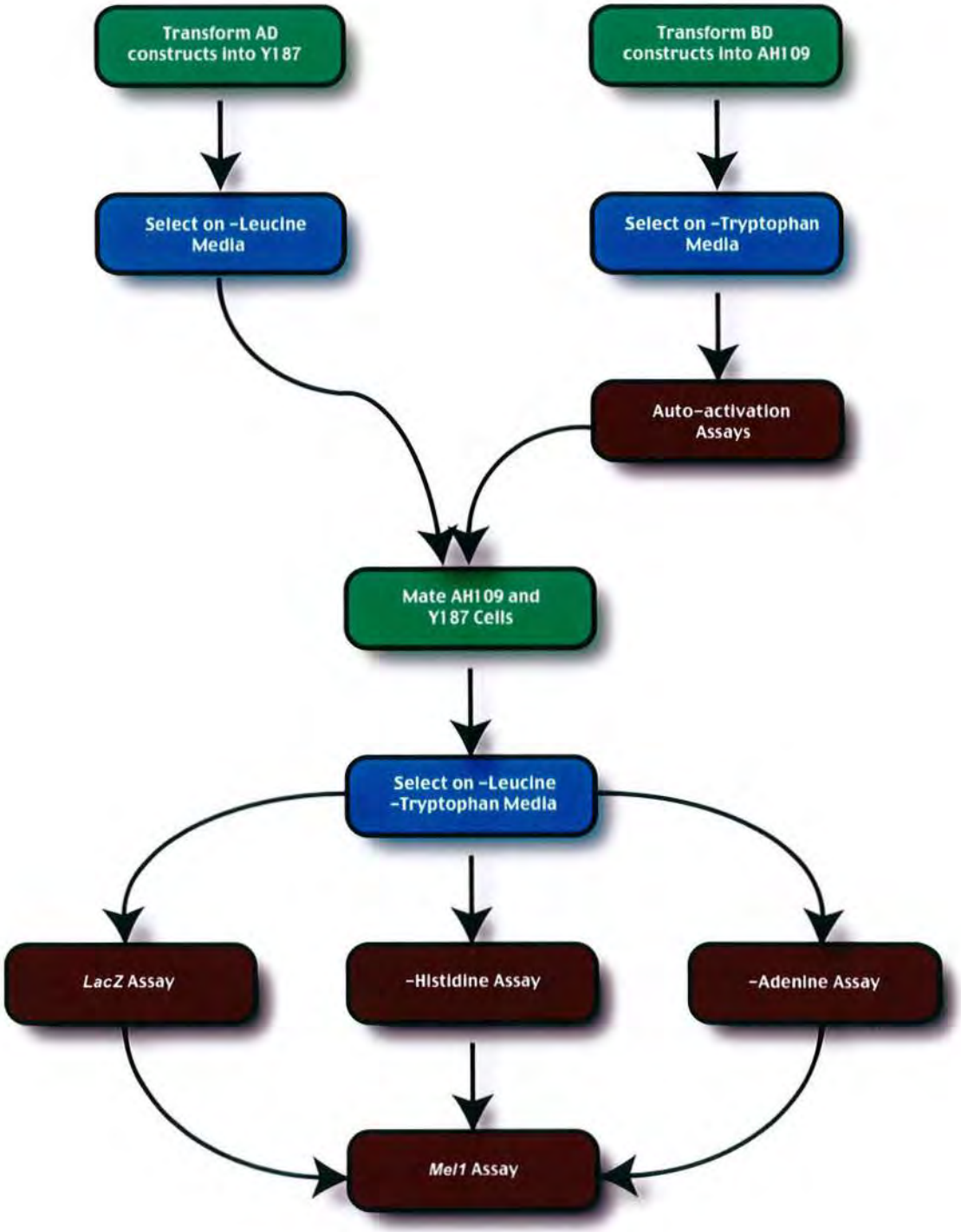


Fig 2.3b. An overview of the reporter genes used in this study, showing the Upstream Activating Sequences and Minimal Promoters.

Figure 2.4: An Overview of the Yeast Two Hybrid Procedure



as it contains the full range of reporters. The transformed yeast were then grown under selective conditions, i.e. upon media lacking Histidine or Adenine. Yeast that did not grow upon this media was deemed to not be demonstrating auto-activation. The *LacZ* reporter gene was also assayed for auto-activation as described in 2.2.6.4

2.2.6.3 Yeast Mating and Selection of Diploids.

Mating was performed using the plate method as described in Causier and Davis (2002). A single colony from the BD vector transformation was re-suspended in 30µL of SDW with 3µL then being transferred onto the surface of a YPD-A plate and allowed to dry. Subsequently a single colony from an AD transformation was also re-suspended in 30µL of SDW, with 3µL then being pipetted onto the BD patch upon the YPD-A plate. The mating was allowed to proceed for 48hrs at 20°C before a scraping was taken from the patch and spread onto a selective plate, i.e. one that lacked both Leucine and Tryptophan. This selects for diploid yeast, as only cells containing both the AD and BD plasmids will be able to overcome the nutritional selection.

2.2.6.4 Identification of Interacting Proteins by Nutritional Selection.

It was hoped that an interaction between the BD and AD-linked fusion proteins of interest would result the formation of a complete *GAL4* transcription factor. This in turn would result in the reporter genes being expressed. Four separate reporters were used; the nutritional genes *HIS3* and *ADE2* (which permit the biosynthesis of Histidine and Adenine) and the visible marker genes *LacZ* and *MEL1* (who's protein-products catalyse the production of a blue precipitate when the correct substrate is added).

Individual colonies were picked from a –Tryptophan –Leucine (-WL) SD plate (and so were therefore assumed to be expressing both proteins of interest) and were re-suspended in 30µL of SDW. They were then transferred onto selective plates by pipetting 3µL onto the surface and allowing the patch to dry before the plate was transferred to 30°C. All of the selective plates lacked Leucine and

Tryptophan (so as to maintain the diploid) but in addition –WLA plates lacked adenine (so as to detect the activity of the *ADE2* gene) and –WLH plates lacked Histidine (to assay for *HIS3* activity).

In addition the yeast were also patched onto a –WL plate containing 40µg ml^{-1} X-α-gal, which is the substrate for the α-galactosidase reporter gene. A blue precipitate would appear upon the surface of the plate with 48hrs if the gene was being expressed.

2.3.6.5 Identification of Interacting Proteins by the LacZ Assay.

The LacZ Assay provided a fourth and final test for protein-protein interactions within yeast. The yeast were grown for 48hrs at 30°C upon a –WL SD plate to maintain a diploid state before a filter (Whatman) was placed upon the surface. The plate was incubated for a further 24 hours to allow yeast to grow into the filter before they were lysed by flash-freezing the filter in liquid N_2 for 5 seconds. The filter was then removed and placed upon another filter that had previously been soaked in 2ml of Assay Buffer (50mM diSodium hydrogen orthophosphate, 50mM Sodium dihydrogen orthophosphate), 10mM Potassium chloride, 1mM Magnesium sulphate, 30mM β-mercaptoethanol and 800µM X-gal. Expression of β-galactosidase results in the production of a blue precipitate within the confines of the lysed yeast spot within 24hours.

Chapter III: An *In silico* analysis of the CAP Family.

3.1 Introduction

The development of Bioinformatics, i.e. the computer-aided analysis of gene and protein function, has provided a rapid method for inferring functional properties based solely upon sequence data. Examples include the use of the Basic Local Alignment Search Tool (BLAST) to compare a known sequence to genomic databases in order to discover homologues and the alignment of multiple sequences in order to identify conserved residues. Computational methods are also commonly used to assess the similarity between related protein sequences and therefore group the proteins into a Phylogenetic tree. Major branches (or clades) are often found to share characteristics and infer evolutionary relationships. In addition Bioinformatic searches may be used to identify certain sequence motifs such as localisation signals, protein or nucleotide binding sites and phosphorylation sites. Such information is often useful in determining the likely functions, methods of regulation and localisation of un-characterised proteins.

Bioinformatics is, in essence, the application of information gathered from prior experimental work to a different problem and as such cannot be considered to be entirely reliable. The results obtained, especially functional predictions, must still be confirmed by actual experimental work. The use of computational analysis can however provide a useful guide and may inform experimental design. These caveats should be taken into account whilst considering the analyses presented in this chapter.

3.2 The CAP Family

A member of the CAP family has been identified within every Eukaryotic kingdom but the CAP of the budding yeast *Saccharomyces cerevisiae* is to date, the best characterised. A list of notable homologues was generated by using the *S.cerevisiae* CAP protein sequence as a search query within the BLAST tool and the resulting matches are presented below in Table 3.1:

Table 3.1 Homologues of the *S.cerevisiae* CAP as Generated by BLAST.

Species	Length (AA's)	Score	Expect	Identity (%)	Positive (%)	Gaps (%)
Candida albicans	545	1161	e^{-125}	44	63	6
Aspergillus nidulans	529	884	$2e^{-93}$	35	56	5
Schizosaccharomyces pombe	551	857	$3e^{-90}$	34	55	8
Dictyostelium discoideum	464	721	$2e^{-74}$	34	54	11
Cryptococcus neoformans	507	786	$6e^{-82}$	35	54	7
Letulina edodes	518	816	$2e^{-85}$	36	56	6
Homo sapiens CAP 2	477	709	$5e^{-73}$	35	53	8
Drosophila melanogaster	528	688	$1e^{-70}$	33	49	7
Danio rerio	463	694	$3e^{-71}$	35	53	6
Xenopus laevis CAP 2	482	728	$3e^{-75}$	34	52	7
Mus musculus CAP 2	476	696	$2e^{-71}$	35	53	8
Caenorhabditis elegans	495	704	$2e^{-72}$	32	53	6
Homo sapiens CAP 1	475	708	$6e^{-73}$	34	52	9
Arabidopsis thaliana	476	457	$8e^{-44}$	26	44	11
Gossypium hirsutum	471	457	$8e^{-44}$	26	43	12
Oryza sativa	492	427	$2e^{-40}$	25	42	9
Mus musculus CAP 1	474	708	$6e^{-73}$	33	51	9
Xenopus laevis CAP 1	475	693	$3e^{-71}$	33	52	12

The BLAST algorithm divides the inputted query sequence into short stretches or 'words' and compares each of these to an extensive sequence database. Matching database sequences will then be compared to neighbouring query words in an attempt to build up a significant alignment. Finally the matching sequences are given a score that is indicative of the degree of similarity between the two sequences and is a product of several factors. These include the number of sequences with a similar or higher score that could have been generated by chance (the Expect value), the number of identical residues (the Identity score), the number of aligned residues with similar properties (the Positive score) and the amount of gaps that were inserted in order to maximise the alignment.

The sequences of the proteins identified by this BLAST search were then aligned using the ClustalX package (Thompson *et al* 1994) and the alignment was imported into the Phylogenetic Analysis Using Parsimony (PAUP) software

(Swofford 2003). This was then used to generate a phylogenetic tree that grouped the CAP family members into clades on the basis of their resemblance to each other. A bootstrap test was then performed which consisted of random point mutations being inserted into the sequences and subsequent recalculation of the tree. This was repeated one hundred times with the effect of the substitutions upon each branches occurrence being recorded. The bootstrap value assigned to each branch reflects the frequency of that branches occurrence and therefore the tolerance of the branch to sequence variation. In effect the bootstrap value represents the statistical significance of the branch. The tree is shown in Figure 3.1.

The phylogenetic tree shows that family members are grouped on the basis of evolutionary lines with the CAPs of higher plants and vertebrates forming distinct groups. There was much less similarity between the fungal CAP's and those of the invertebrates. Interestingly the vertebrate CAP2 isoforms formed a group of their own which demonstrates that they have a greater similarity to each other than they do to their respective species CAP1 isoform. This is strongly suggestive of there being functional differences between the CAP1 and 2 isoforms.

A further alignment was performed using several key members of the CAP family (i.e. those that have been functionally or developmentally characterised) and this is shown in Figure 3.2. It can be seem that there are many short sequences that are highly conserved including the N-terminal RLE repeats that are hypothesised to be involved in Adenylate cyclase binding (Hubberstey and Mottillo 2002). In addition the P1 Proline-rich domain is also highly conserved which suggests that the interaction between CAP and Profilin is evolutionarily conserved. Further highly conserved regions include some of the residues previously shown to be essential for G-actin binding (Zelicof *et al* 1994) and the WH2/VH domain. The later example is interesting given that the WH2/VH domain of CAP has been shown to be unable to bind actin (Mattilla *et al* 2004); it presumably fulfils some other purpose. The P2 Proline-rich domain however is almost entirely absent from the non-yeast CAPs which suggests that they do not interact with SH3 domain-containing proteins via a Proline-rich region.

Fig 3.1: A Phylogenetic Tree of the CAP Family.

Fig 3.1: A rectangular cladogram of the CAP family from a diverse group of Eukaryotes shows four major groups of interest. Conserved differentiaion between type 1 and type 2 isoforms results in the higher animal CAPs forming two distinct groups. The higher plant CAPs appear to be closely related and so form one group with some similarity to Dictyostelium. Finally it appears that there is great diversity amongst the Fungal CAPs.

The Bootstrap values printed on each node represents the percentage occurance of the branch when random substitutions were made to the protein sequences. They therefore represent the statistical confidence in each node.

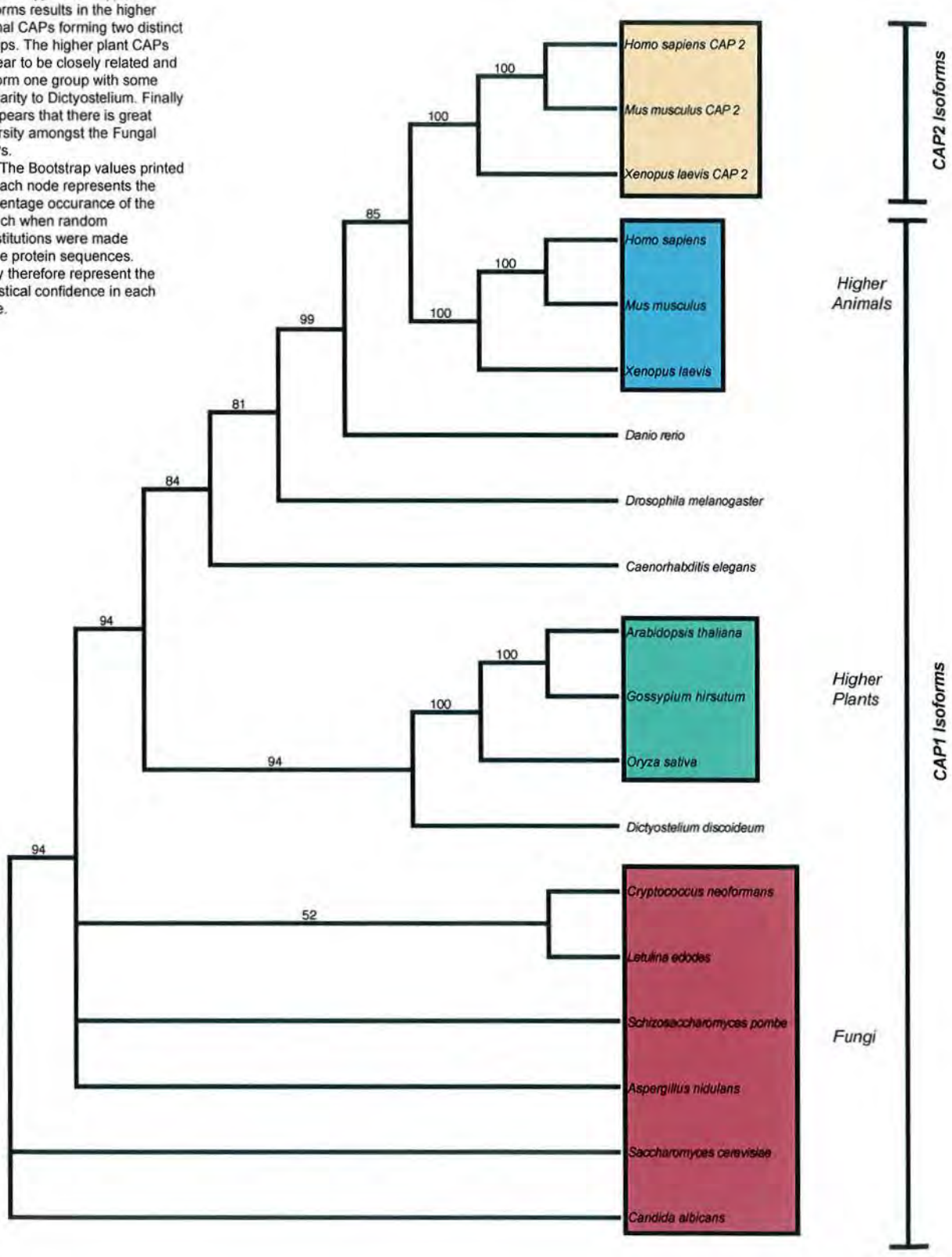


Figure 3.2: An Alignment of CAP Family Members

Fig3.2: A representitive selection of the characterised CAP family members were aligned using the ClustalX package. Conserved residues are highlighted in blue. It can be seen that the N-terminal RLE repeats (which are thought to interact with adenylate cyclases) are highly conserved as are certain residues within the P1 Poly-Proline domain. There are also a number of conserved residues throughout the Carboxyl-terminus, which may play some role in the CAP/G-Actin interaction. The residues indentified as being important for Actin binding by Mattila *et al* (2004) are shown in green; these demonstrate some degree of conservation.

3.2.1 Several Higher Plant Species Possess CAP Homologues.

A search of genomic databases revealed three full-length higher plant CAP family members, the previously characterised Arabidopsis (Barrero *et al* 2002) and Gossypium CAPs (Kawai *et al* 1998), and a rice (*Oryza sativa*) homolog. In addition a search of an Expressed Sequence Tag (EST) database returned matches from *Solanum tuberosum* (Potato), *Lotus japonicus* (Lotus), *Glycine max* (Soybean), *Medicago truncatula* (a legume), *Triticum aestivum* (Wheat), *Populus tremula* (a Poplar Tree) and *Zea mays* (Maize). Therefore CAP is widely dispersed amongst the higher plants.

An alignment between the three full-length plant CAPs and the substantial *Zea mays* EST is shown in Figure 3.3; the three four proteins exhibit a high degree of similarity. Both the candidate Adenylate cyclase-binding domain and the P1 domain are conserved. The high degree of homology is highly suggestive of functional conservation between the Higher Plant CAPs.

3.3 An Analysis of Arabidopsis thaliana CAP1

A BLAST search of the complete *Arabidopsis* genomic sequence with the AtCAP1 (At4g34490) gene as a query revealed that AtCAP1 is a single copy gene, i.e. there were no close matches (beyond the AtCAP1 gene itself) returned. The results are shown in Table 3.2 below:

Table 3.2 Closest Matches to AtCAP1 in the *Arabidopsis* Genome.

GENE	SCORE	EXPECT
AtCAP1	949	0
CLP Protease Subunit	75	0.27
Calmodulin Binding Protein	72	0.6
Cyclin Dependant Kinase	70	1.0

It can be seen that there are no close homologues present and therefore it may reasonably be assumed that AtCAP1 is the sole CAP family member present in *Arabidopsis*. A comparison between AtCAP1 and *S.cerevisiae* CAP (shown in Figure 3.4) shows that several of the functional regions are conserved. In particular both RLE motifs are present in the Arabidopsis protein

Figure 3.3: An Alignment of Plant CAP Family Members.

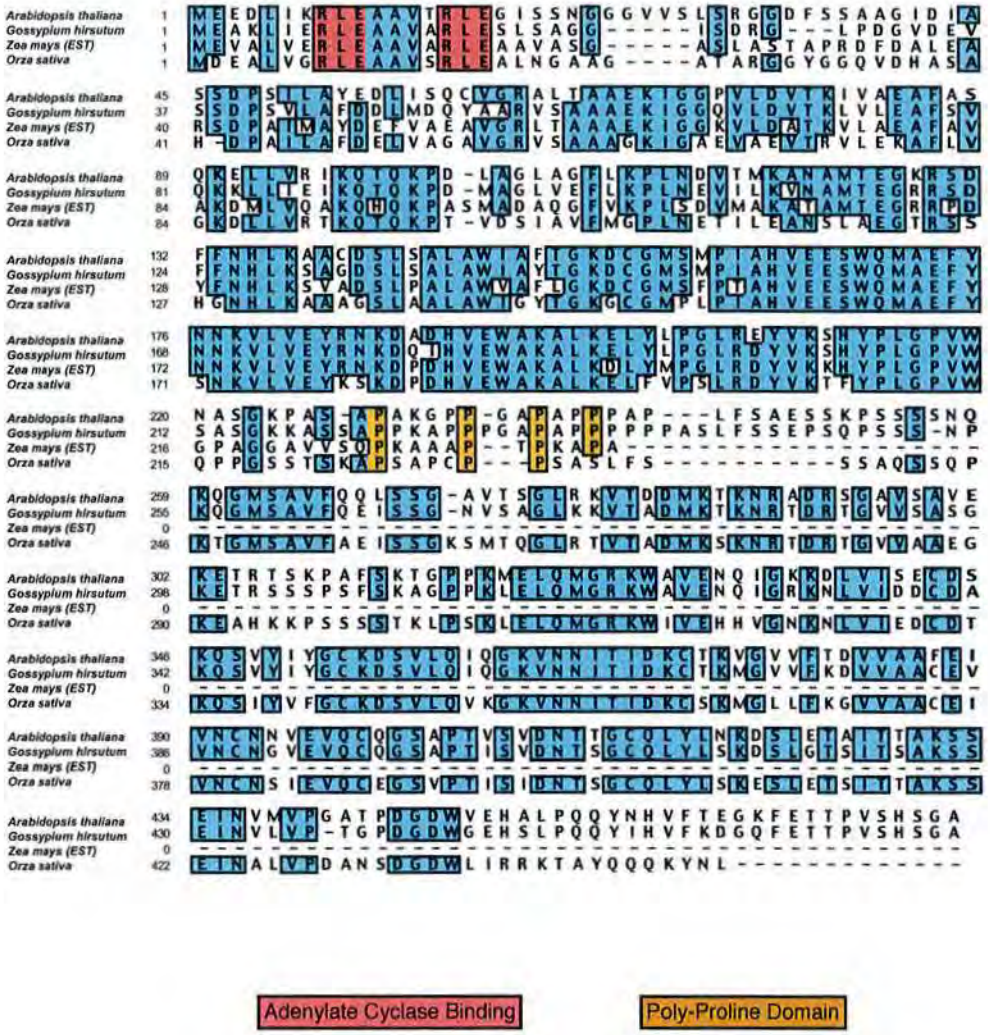


Fig 3.3: Analysis of higher plant genomes revealed three complete CAP family members. In addition a substantial Maize EST was identified. The four proteins show a high degree of similarity with the Adenylate cyclase -binding RLE repeat being conserved. In addition four residues are preserved within the Poly-Proline domain.

Figure 3.4: A Comparison of the *Arabidopsis* and *Saccharomyces* CAP's

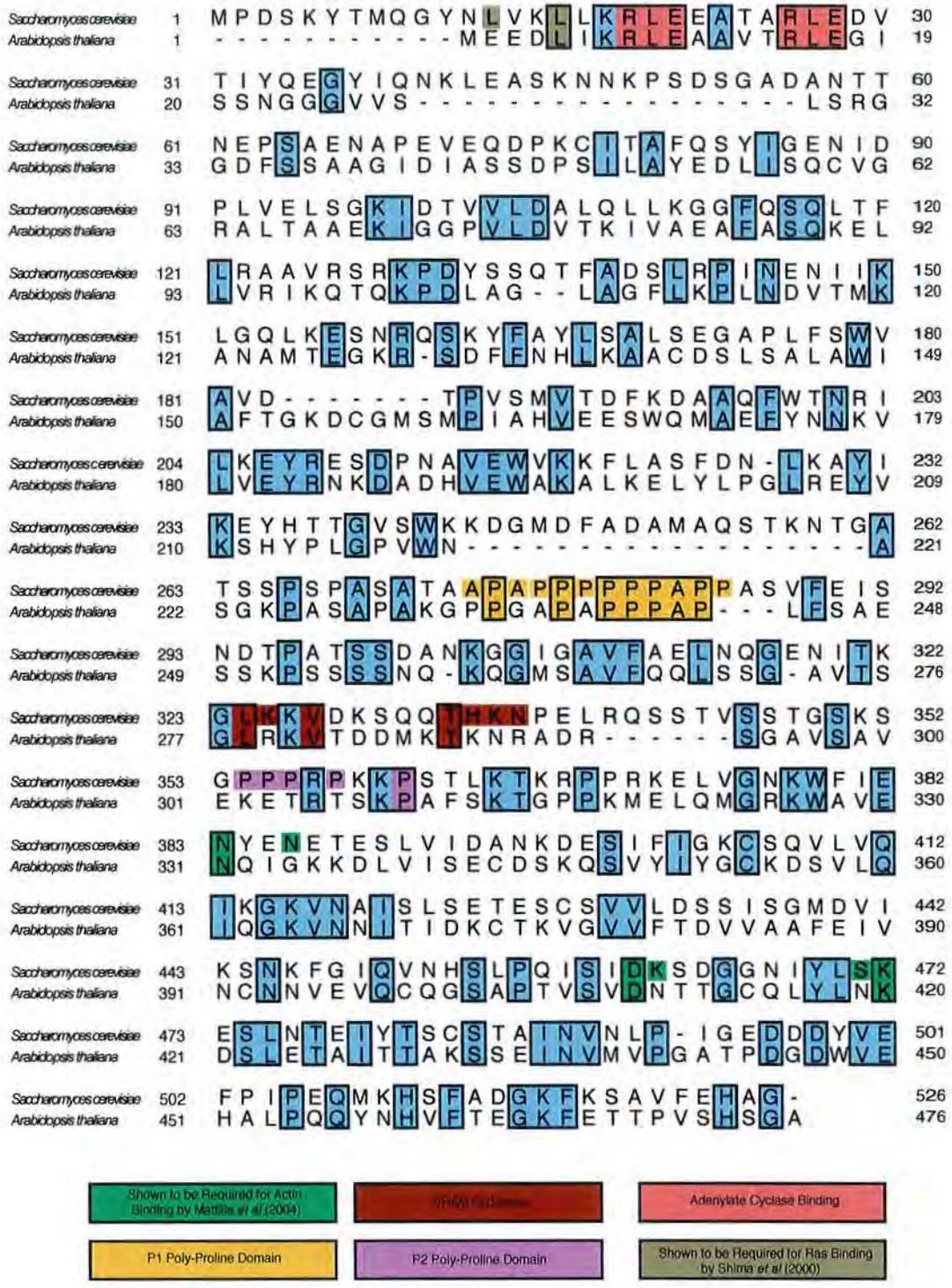


Fig 3.4: Both proteins include the conserved RLE repeats, which are thought to interact with Adenylate cyclases and a substantial P1 Domain, which is believed to bind Profilin. The *Arabidopsis* protein however lacks a P2 domain and most of the WH2/VH domain. In addition several of the residues shown to be essential for Actin binding are absent as is one of the Ras interacting points. Un-boxed highlighted residues are those that are believed to be involved in interactions but are not conserved between the two proteins.

and the P1 domain is mostly intact. The relevance of the RLE motifs is unclear as interactions between CAP and Adenylate cyclases are yet to be shown to occur outside of the yeast model and a clear candidate for a conventional membrane-bound higher plant Adenylate cyclase is yet to be identified. The presence of the P1 domain makes an interaction with Profilin likely although experimental evidence would be required to confirm this. The conservation of Leucine[15] in the *Arabidopsis* protein is unlikely to be of importance as the Arabidopsis genome lacks a Ras homologue and the Adenylate cyclases of higher Eukaryotes are not believed to be activated by Ras.

The lack of the second Proline-rich domain (the P2 domain) is indicative of an inability to interact with SH3-domain containing proteins. The sole function attributed to the P2 domain of *S.cerevisiae* CAP is the interaction with ABP1p (a protein that *Arabidopsis* lacks) via it's P2 domain, so the absence of this domain from the *Arabidopsis* homologue is unsurprising. The lack of a complete WH2/VH domain is also unlikely to have a functional consequence as this domain has been shown to be unable to bind monomeric actin in the *S.cerevisiae* CAP homologue (Mattilla *et al* 2004).

Examination of the aligned Carboxyl termini reveals that some of the residues shown to be essential for actin binding by Mattilla *et al* (2004) are conserved. The exact nature of the actin binding site is unclear as a separate study (Zellicof *et al* 1996) showed that the final 27 amino-acid residues of *S.cerevisiae* CAP were required to interact with G-actin. Therefore it is difficult to draw a conclusion regarding the ability of AtCAP1 from the alignment and degree of conservation.

3.3.1 The Predicted Cellular Localisation of AtCAP1

Protein function is at least partially governed by it's localisation as this determines both the proteins with which it may interact and the environmental conditions, such as pH or ionic concentration, in which it must operate. Localisation is often a result of targeting sequences within a protein; these are conserved and therefore a proteins eventual destination may be predicted. The TargetP algorithm (Emanuelson *et al* 2000) predicts that AtCAP1 is not

localised to Mitochondria, Chloroplasts or the Secretory Pathway. The results of the analysis are shown below in Table 3.3

Table 3.3: TargetP Predictions for AtCAP1 Localisation

ORGANELLE	PROBABILITY
Mitochondrion	0.114
Chloroplast	0.069
Secretory Pathway Component	0.097
Other	0.883

The application of the Psort algorithm (Nakai and Kanehisa 1992) to AtCAP1 yields a similar result although the program is more specific in that it predicts a cytoplasmic localisation (presumably due to the absence of a nuclear targeting signal). The results of the analysis is shown in Table 3.4 below:

Table 3.4: PSort Predictions for AtCAP1 Localisation.

ORGANELLE	PROBABILITY
Cytoplasm	0.650
Mitochondrial Matrix Space	0.100
Chloroplast Thylakoid Membrane	0.100
Endoplasmic Reticulum	0.000

3.3.2 Candidate Phosphorylation Sites

Phosphorylation is an important method of functional regulation and is mediated by the addition of a phosphate group to a target protein. Phosphorylation sites consist of a Tyrosine, Threonine or Serine residue flanked by conserved residues. The composition of the phosphorylation site determines the class of kinase that is able to recognise and phosphorylate it, therefore phosphorylation sites may be predicted. The application of the Eukaryotic Linear Motif (ELM) software (Puntervoll *et al* 2003) to AtCAP1 generated

candidate phosphorylation and regulatory sites and these are shown in Table 3.5 below:

Table 3.5: ELM Predicted Phosphorylation Sites

SEQUENCE	POSITION	DESCRIPTION
KELLV	90-94	Cyclin Binding Site
KVLV	178-181	Cyclin Binding Site
KELYL	198-202	Cyclin Binding Site
KDLVI	336-340	Cyclin Binding Site
DIASSDPS	42-49	GSK3 Phosphorylation Site
PLFSAESS	243-250	GSK3 Phosphorylation Site
SAESSKPS	246-253	GSK3 Phosphorylation Site
AESSKPSS	247-254	GSK3 Phosphorylation Site
QLSSGAVT	268-275	GSK3 Phosphorylation Site
ADRSGAVS	291-298	GSK3 Phosphorylation Site
LVISECDS	338-345	GSK3 Phosphorylation Site
KFETTPVS	465-472	GSK3 Phosphorylation Site
RKVTDDM	279-285	PKA Phosphorylation Site
TRTSKPA	304-310	PKA Phosphorylation Site
PGATPDG	440-446	Proline-Directed MAP Kinase Phosphorylation Site
FETTPVS	466-472	Proline-Directed MAP Kinase Phosphorylation Site

Further stringency was applied by utilising a second search technique, i.e. the NetPhos Package (Blom *et al* 1999). The data generated by that search is shown below in Table 3.6 (only sites with a phosphorylation probability in excess of 0.5 are shown).

Table 3.6: NetPhos Predicted Phosphorylation Sites.

PHOSPHORYLATED RESIDUE	POSITION	NETPHOS SCORE
Serine	30	0.911
Serine	49	0.708
Threonine	66	0.903
Threonine	99	0.955
Serine	130	0.805
Threonine	152	0.685

Serine	168	0.787
Serine	222	0.818
Serine	246	0.890
Serine	249	0.953
Serine	250	0.532
Serine	253	0.937
Serine	254	0.996
Serine	298	0.994
Threonine	306	0.935
Serine	307	0.983
Serine	312	0.982
Serine	341	0.994
Serine	407	0.792
Tyrosine	417	0.536
Threonine	429	0.850
Serine	432	0.985
Serine	433	0.823
Threonine	443	0.949
Threonine	469	0.944
Serine	472	0.913

There is great disparity between the results generated by the two methods but some candidate sites were selected by both and shall be considered further. Glycogen Synthase Kinase 3 (GSK3) is an enzyme that has been implicated in the control of the insulin response in animal systems via the phosphorylation of metabolic enzymes (McManus *et al* 2005). It seems unlikely that the phosphorylation of AtCAP1 would play a role in such a signaling pathway but several of the candidate GSK3 sites were identified by both search methods (i.e. those at positions 49, 250, 253, 254, 298, 341 and 469) and so must be considered. It is interesting to note that the higher plant GSK3 homologues have previously been shown to be involved in the response to osmotic stress (Piao *et al* 1999); a process in which the involvement of Actin and therefore AtCAP1 could easily be conceived.

The potential cAMP-dependant Protein Kinase (PKA) phosphorylation site at position 307 is interesting given the direct involvement of CAP in the production of cAMP (in yeast) and the stimulatory effect of cAMP upon PKAs activity. The phosphorylation of CAP by PKA followed by a reduction of cAMP production would provide a mechanism by which cAMP signaling could be regulated on a negative-feedback basis. This of course assumes that AtCAP1 is involved in the production of cAMP. Proline directed kinases phosphorylate positions adjacent to Proline residues and include the Mitogen Activated Protein

(MAP) Kinases family which are key regulators of cell division. The candidate sites at positions 443 and 469 were identified by both searches and would be expected that a protein involved in the regulation of the Actin cytoskeleton would itself be tied to cell-cycle control mechanisms such as MAP Kinases.

3.4 The Predicted Expression Pattern of AtCAP1

The use of DNA-microarray technology allows the entire transcriptional program of a specific tissue to be analysed and compared to that of other samples. This is particularly useful for comparing the gene expression programs of different developmental stages or in identifying tissue-specific genes. In addition changes in expression induced by exogenous biotic and abiotic stresses may also be studied. The analyses presented here were performed by using the GeneSwinger tool provided by the National Arabidopsis Stock Centre's (NASC) coupled with a database of results achieved using the Affymetrix ATH1 microarray; this gene-chip includes sequences corresponding to approximately 24.000 distinct *Arabidopsis thaliana* genes. The GeneSwinger utility searches for the experiments that demonstrated the greatest variation of a chosen genes expression levels. The use of AtCAP1 (At4g34490) as a search term returned several experiments relating to pollen development. Firstly an experiment performed by the ATGEN consortium (within the laboratory of David Twell) showed that the expression of AtCAP1 in mature pollen grains was high in comparison to that shown by complete flowers (a raw signal strength of 1138.07 was observed in the pollen as opposed to 273.55 in the flower). These values are the mean average of three replicates and have been normalised to allow non gene-specific sample variations to be discounted and so to permit valid comparisons to be made. Such an apparent enrichment of CAP could be a product of globally increased gene expression within pollen as opposed to a increased requirement for CAP. This possibility was discounted by examining the expression pattern of the DNA Polymerase complexes ∂ subunit (At2g29570), which is a typical housekeeping gene. In fact it's expression was found to be significantly lower in mature pollen grains than in the flower as a whole (27.68 as opposed to 319.99). This is shown graphically in Figure 3.5a.

A further study performed by Twell *et al* (unpublished data) and included in the NASC database monitored the expression of genes throughout pollen

Figure 3.5: Micro-Array analysis of AtCAP1 Expession

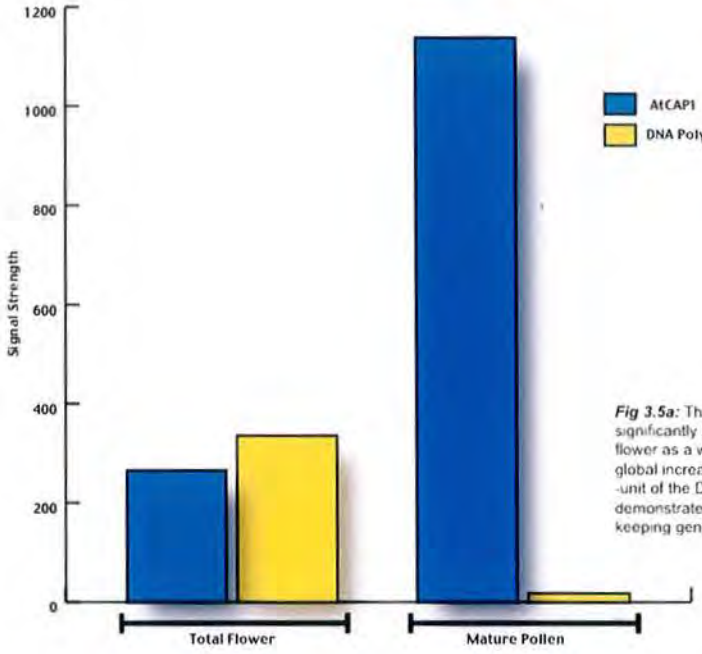


Fig 3.5a: The expression of AtCAP1 appears to be significantly higher in Mature Pollen than in the flower as a whole. This is not the result of a global increase in gene expression as the ∂ sub -unit of the DNA polyermase complex does not demonstrate such an increase. Other house keeping genes also show such a decrease

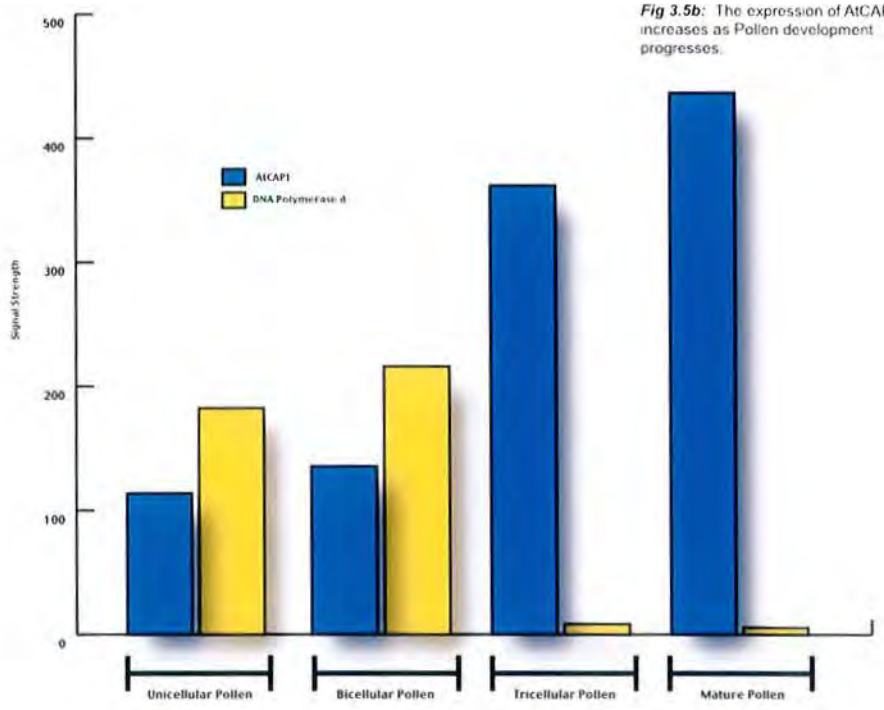


Fig 3.5b: The expression of AtCAP1 increases as Pollen development progresses.

development. The results of this experiment are shown below in Table 3.7 and graphically in Figure 3.5b. Again this data is the mean of several replicates and has been normalised to allow meaningful comparisons to be made between slides.

Table 3.7: The Expression of AtCAP1 During Pollen Development.

	Unicellular Pollen	Bicellular Pollen	Tricellular Pollen	Mature Pollen
AtCAP1	114	136	362	437
DNA Polymerase ∂	182	216	8	6

It can be seen that the expression of AtCAP1 increases as pollen development progresses with an especially large increase coinciding with the onset of the tri-cellular stage. The expression of the comparison house-keeping gene (DNA Polymerase ∂) decreases as time progresses which again indicates that AtCAP1 bucks the global trend.

3.5 Chapter Discussion

Bio-informatics is by its very nature an inexact science but it may be used to generate a broad profile of a genes function prior to its experimental characterisation. This assertion and the analyses presented in this chapter allow several broad predictions regarding AtCAP1's functionality to be made. It can be stated with a reasonable degree of confidence that AtCAP1 is sole *Arabidopsis* CAP gene and as a single copy gene is likely to be functionally important. Other members of the CAP family are believed to be primarily cytosolic (although they may be recruited to the membrane via an adenylate cyclase interaction). This and the lack of organelle targeting sequences within AtCAP1 suggests that it too is a cytosolic protein. This is what would be expected for an actin interacting protein.

The conserved similarities between AtCAP1 and other members of the CAP family suggest some degree of functional conservation with the alignment between AtCAP1 and *S.cerevisiae* CAP (Figure 3.4) being of particular interest. The RLE motifs at the N-terminus of *S.cerevisiae* CAP are believed to be involved in the interaction between CAP and the CYR1 adenylate cyclase

(Hubberstey and Mottillo 2002) and are conserved in most known CAP homologues including AtCAP1. This is puzzling given that none of the higher Eukaryotic CAP family members have been shown to directly interact with an adenylate cyclase although one possibility is that these residues are involved in CAP's dimerisation which is also (partially) an N-terminal function (Hubberstey and Mottillo 2002).

Half of the residues shown to be essential for actin monomer binding by Mattilla *et al* (2004) are conserved in AtCAP1 as are several residues within the final 27 amino acids, which has also been shown to be involved in actin binding (Zelicof *et al* 1996). The exact nature of the G-Actin binding domain is still unknown so it is difficult to make a prediction regarding the ability of AtCAP1 to bind Actin but the conservation of these key residues raises this as a possibility. There is a high degree of conservation between the two proteins Proline-rich P1 division which is suggestive of an interaction between AtCAP1 and members of the Profilin family as has been observed for other CAP's (Hubberstey and Mottillo 2002). It is however unlikely that AtCAP1 promotes actin dynamics via interacting with Profilin (as has been proposed for other CAPs) as plant Profilins are not believed to induce monomer nucleotide exchange (Perelroizen *et al* 1996b).

The potential phosphorylation sites identified by these analyses are of interest as they allow possible regulatory mechanisms to be determined. The candidate PKA phosphorylation site (position 307) is especially interesting as it raises the possibility that AtCAP1 may be regulated by the cell's cAMP levels. A potential negative-feedback regulatory mechanism where an excess of cAMP leads to PKA activation which in turn results in the phosphorylation of AtCAP1 and the subsequent cessation of cAMP production may be imagined. The phosphorylation of AtCAP1 would in addition permit the binding of a 14-3-3 protein as has been shown to occur to a fungal CAP (Zhou *et al* 2000) and could form the basis of another form of regulation.

It would be expected that a protein that plays an important role in regulating the actin cytoskeleton would be of great importance in mature pollen grains as the germination and growth of a pollen tube is dependant upon dynamic actin (see Section 1.3.4). It therefore of little surprise to find that mature pollen grains are, in comparison to other floral tissues, enriched for AtCAP1. This is shown by the dramatic disparity in expression levels between

the two tissues. The increase in AtCAP1 expression throughout pollen development does not necessarily indicate that CAP is required for the development of the gamete; it is equally likely that CAP is being produced in preparation for germination.

The ideas presented in this Chapter were generated by the use of computer software and are an attempt to discern some of AtCAP1s functional characteristic based solely on its sequence and similarity to previously characterised proteins. As such the results presented here are not substantiated by experimental evidence and should be considered as being more akin to estimates than measured data. The results of this analysis do however grant a useful insight into the potential function of AtCAP1 and have informed the experiments presented in the following chapters.

Chapter IV: AtCAP1 in Higher Plant Development.

The main objectives of the work described in this Chapter were to:

I *Assess the effect of eliminating AtCAP1 expression upon Arabidopsis thaliana morphology and development*

II *Examine the interaction between CAP family members and the Actin cytoskeleton in gametes*

III *Investigate the effect of eliminating AtCAP1 expression upon intra-cellular Actin organisation*

IV *Attempt to differentiate between phenotypes caused by a disruption to the Actin cytoskeleton and those induced by the loss of cAMP signalling*

4.1 Introduction

The elimination or reduction of a gene of interests expression is commonly used to investigate the *in vivo* function of that gene. Phenotypic differences between the modified plant and wild-type examples, which are presumed to include a functional copy of the gene, are often the desired goal of the investigation. The work presented in this chapter consists of an analysis of an *Arabidopsis thaliana* line with a disrupted AtCAP1 locus. This disruption is caused by the insertion of an *Agrobacterium tumefaciens* derived tDNA fragment into the genome with the result being the elimination of the genes transcription and therefore expression of the protein. The *Agrobacterium* used for these transformations are non-virulent as they lack the Ti plasmid; therefore tumours are not induced and any identifiable phenotypes may be assumed to be due to the disruption of the genomic sequence. The location of tDNA insertion is a largely random and so must be identified in order for lines with

Figure 4.1: The Locations of T-DNA Insertions Within AtCAP1

Fig 4.1:The T-DNA insertions used in this study are distributed throughout the genomic sequence. Syngenta 635-A5 is located within the promoter sequence whereas the SALK and GABI-Kat T-DNA's are inserted within exons (Shown in yellow).

insertions in genes of interest to be identified. This is most often achieved by sequencing the region flanking the tDNA and aligning this to the genomic sequence. The flanking region will have been amplified by the use of the Polymerase Chain Reaction (PCR) and a primer specific to the border of the tDNA. The generation of large easily available collections of these insertion mutants has been invaluable to plant research as it allows the individual researcher to study the effects of gene knockout without having to perform an extensive mutant screen.

The analyses of these artificially created mutants can lead to the elucidation of the disrupted genes function and may include anatomical, physiological and biochemical studies. The work presented in this chapter represents an attempt to study the effect of AtCAP1's disruption upon the development of a higher plant (*Arabidopsis thaliana*) by identifying a CAP-deficient mutant and analysing its anatomical and physiological characteristics. Particular attention is paid to the development and elongation of its root hairs and to the viability of its pollen.

4.2 The Identification of Knockout Lines.

A search of mutant databases revealed three *Arabidopsis* lines with tDNA insertions proximal to the AtCAP1 locus; these being Syngenta 635 A5 (collection described by Sessions *et al* 2002), SALK 112802 (collection described by Alonso *et al* 2003) and GABI-KAT 453 (collection described by Rosso *et al* 2003). The positions of the three insertions are shown in Figure 4.1, it can be seen that the left borders of the tDNA SALK 112802 and GABI-KAT 453G08 are within exons whereas Syngenta 635 A5 is predicted to lie 300 base-pairs upstream of AtCAP1s start codon. It was believed that the SALK and GABI-KAT insertions would lead to either the total elimination of expression or at the least the production of a truncated transcript. In contrast it was believed that the Syngenta insert would disrupt the promoters and so may lead to a reduction of expression (due to the interruption of enhancer elements) or again the total elimination of expression (due to either the disruption of essential promoter elements or the destabilisation of the transcript).

Seeds from all three lines were plated out as described in Section 2.2.2.1 and were cultivated prior to DNA extraction (as described in Section

2.2.2.2). Samples were genotyped by the method described in Section 2.2.2.3 and shown in Figure 2.2; in essence the plants were characterised using two separate PCR reactions. The production of a PCR product by a reaction containing a primer specific to the left border of the tDNA was indicative of the presence of the insert whereas the production of a product from a reaction containing primers designed to amplify the region encompassing the insertion site was indicative of an uninterrupted wild-type allele. The objective was to identify plants that were homozygous for the insertion as these will lack a functional copy of the AtCAP1 gene. Additionally a PCR product generated by the insert-specific reaction was sequenced in order to confirm the location of the insertion. All three inserts were found to be located within their predicted regions.

4.3 The Effect of the Insertions upon Plant Morphology.

The roots of plants homozygous for the Syngenta 635 A5 insertion were not found to significantly differ from wild-type plants that were grown under identical conditions upon adjacent plates. This statement is based upon a comparison of general morphology between the two plates with factors such as root length, width, growth rate and appearance being considered. The size and positioning of root hairs was also compared. Initial studies upon the GABI-KAT 453G08 insertion line also revealed no discernable phenotype but later work with a larger population (performed by Dr. Mike Deeks) detected a phenotype comparable to that of the SALK 112802 line that is described below.

Plants homozygous for the SALK 112802 insertion were found to lack the characteristic collar of root hairs that develops around the emerging primary root following germination. This phenotype is apparent at two days post-germination and examples of the both the mutant and a plant azygous for the insertion (the Azygote includes two copies of the wild-type allele) are shown in Figure 4.2. The azygotes were used as a control for these comparisons as they would be expected to share any additional (i.e. non-CAP) insertions with the homozygote whereas wild-type plants would not. In doing so the probability of the observed phenotypes being a product of the disruption of other genes was reduced. The absence of the root hair collar can clearly be seen in the figure and this characteristic has been found in all homozygotes examined to date (i.e.

Figure 4.2: The Knockout of AtCAP1 Disrupts Root Hair Collar Development

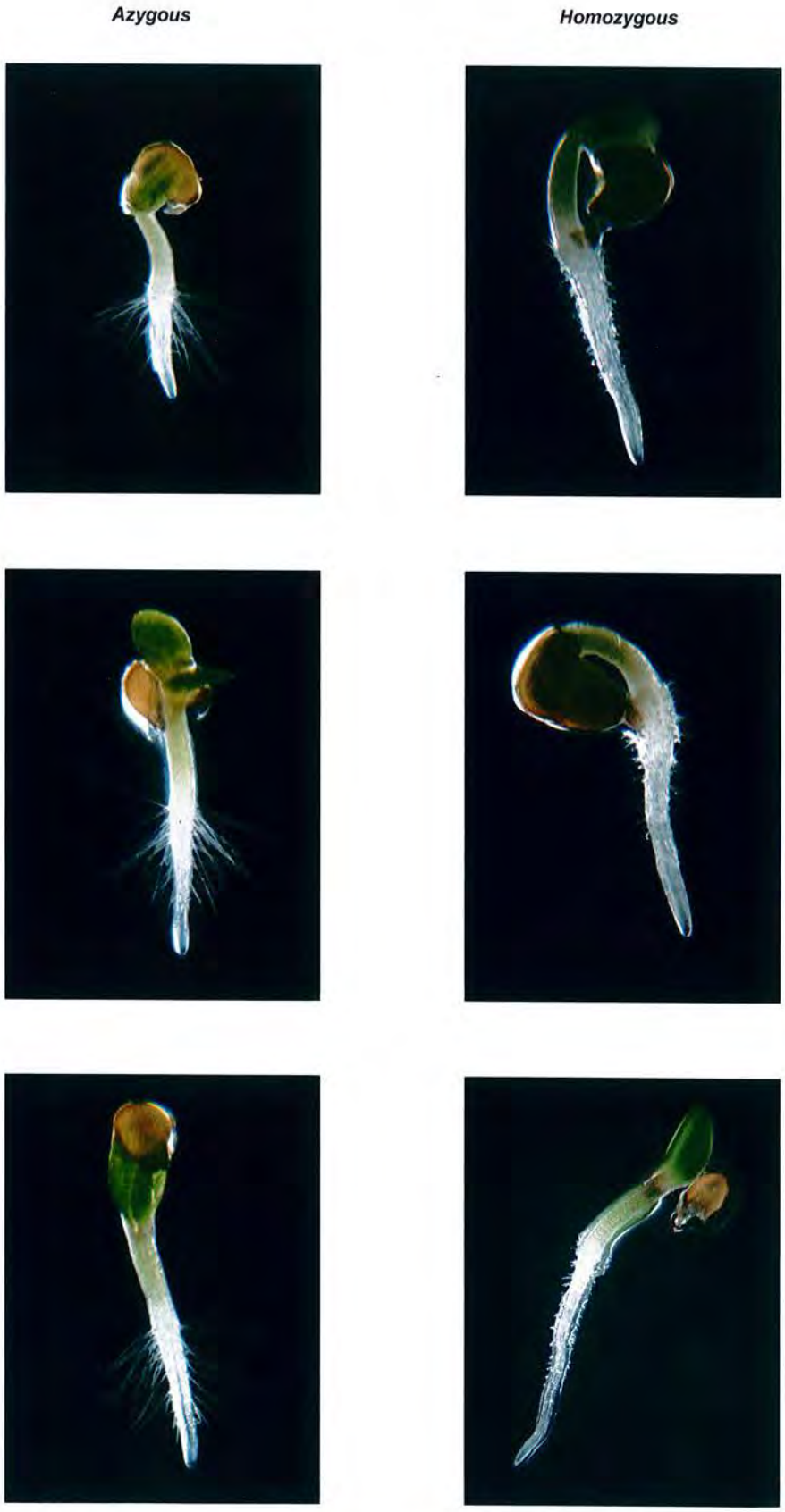


Fig 4.2: Plants homozygous for the SALK 112802 insertion lack the characteristic collar of root-hairs surrounding the base of the developing root at 2 days post-germination. This phenotype is not visible in the Azygotes.

the penetrance was absolute). This phenotype has also been shown to persist through three generations of *Arabidopsis* and always segregates with the insertion.

Examination of the SALK 112802 homozygotes further in development revealed a further root hair phenotype; this is illustrated in Figure 4.3. In effect it appears that the root hairs fail to extend although small 'bumps' can clearly be seen along the surface of the root suggesting that root hair initiation is not totally impeded. This phenotype was observed along the length of the root and the images shown in Figure 4.3 are representative of this.

A statistical analysis of root development was performed in an attempt to better characterise the SALK 112802 phenotype. Initially the growth of the primary root was measured and a comparison between an azygous and homozygous population made; the results of this are shown below in Table 4.1 and are presented graphically in Figure 4.4a:

Table 4.1: The Growth of the Primary Root.

	Time Post Transfer (Days)				
	3	4	5	7	Mean Root Length (mm)
Azygotes	2.45	4.80	8.43	16.42	
Homozygotes	3.72	6.14	9.84	20.17	
Difference (%)	51.8	27.9	16.7	22.8	

It can be seen that the roots of plants homozygous for the insertion were on average longer than azygotes, although this difference does not increase as development progresses. This difference was greater than the standard deviation associated with each sample (as represented by the error bars in Fig 4.4a) A paired t-test was performed in order to further assess the statistical relevance of the observed difference. It was found that the probability of the difference being due to chance was 0.0297 (when 95% confidence intervals were used). This is a reasonably high value and it makes it impossible to confidently say that there was a significant difference in root length.

The growth of individual root hairs was also measured and again there was found to be a significant difference between the two populations. This is shown below in Table 4.2 and the result is graphically represented by Figure 4.4b.

Figure 4.3: The Knockout of AtCAP1 Results in a Root Hair Phenotype

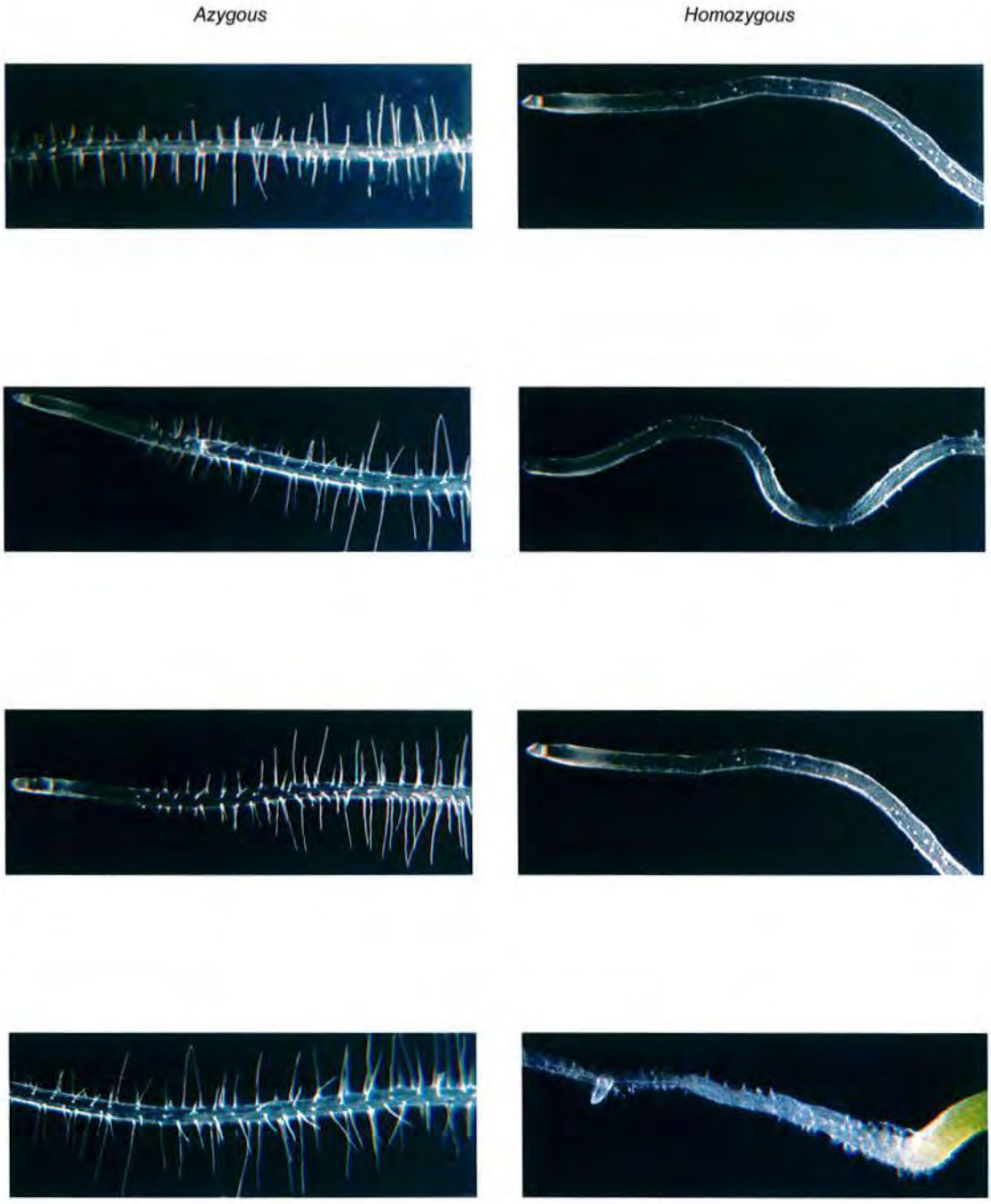


Fig 4.2: Plants homozygous for the SALK 112802 insertion show a dramatic Root-Hair phenotype. Extension of the hairs appears to be severely impaired along the entire length of the root. The images shown here were taken 8 days post-germination.

Table 4.2: Length of Root Hairs

	Mean Root Hair Length (mm)		
Azygotes	0.381	Standard Deviation	0.158
Homozygotes	0.081		0.026
Difference (%)	78.74		

It can be seen that there is a significant difference between the two populations although there was an enormous degree of variation within the azygous sample alone. This results in a large standard deviation which represents 41% of the mean root hair length. The mean root hair length of the homozygotes is however less than the range observed within the azygous population and so the difference between the two samples is considered to be significant. The diverse length of the Azygous root hairs is typical of wild-type *Arabidopsis* and is partially a result of sampling along the entire root length. A paired t-test returned a value of 0.000005, meaning that it is extremely unlikely that such a variation could be caused by random variation.

The density of visible root hairs is indicative of root hair initiation and so the frequency of root hairs within several 3 mm segments of both homozygous and azygous root was counted. The results are shown below in Table 4.3 and are presented graphically in Figure 4.4c.

Table 4.3: The Density of Root Hairs

	Population	
	Azygotes	Homozygotes
Frequency of Root Hairs Segment $^{-1}$	41.33	26.17
Standard Deviation	4.68	3.08
Frequency of Root Hairs mm^{-1}	13.78	8.72
Difference (%)	36.72	

Again there is a difference between the two populations and again this difference in excess of their own variation suggesting that it is a result of the AtCAP1 locus being disrupted. A paired t-test returned a value of 0.0002 which again is suggestive of there being a significant difference between the

Figure 4.4: The Development of Root Hairs in CAP-deficient *Arabidopsis*.

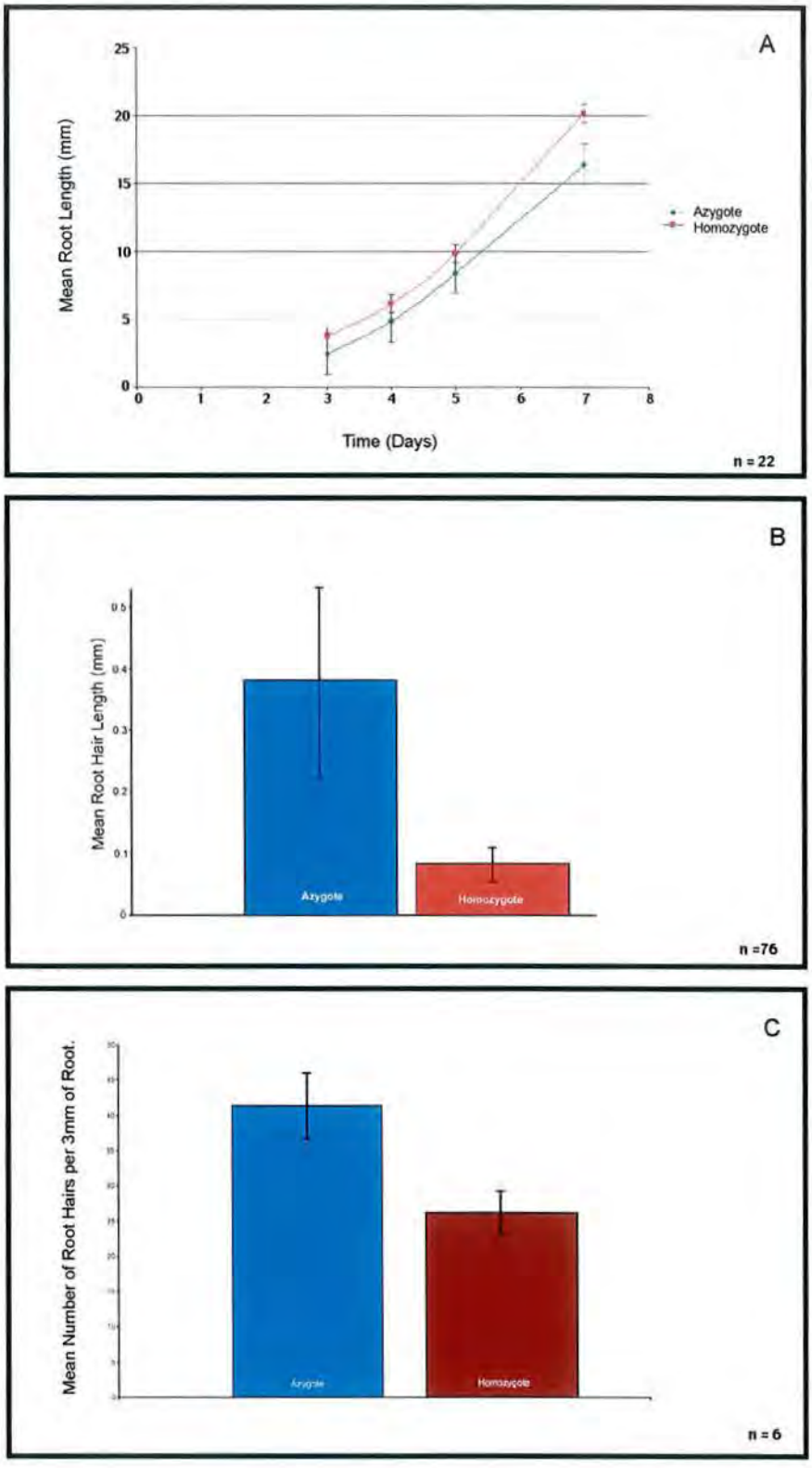


Fig 4.4: The lack of a functional AtCAP1 gene results in both reduced Root Hair initiation and expansion (B and C) although there is relatively little effect upon overall root growth (A). The root hair length and density measurements were taken 8 days post germination and were representitive of sections along the length of the root. The Error Bars on all three panels represent the Standard Deviation of the data Set.

populations (i.e. the variation is unlikely to have been caused by random chance).

The difference between the homozygous and azygous populations persisted once the plants had been transferred onto soil. A delay in bolting was observed; Figure 4.5 shows homozygous and azygous plants approximately two weeks after transplantation onto soil and a clear difference in size may be seen. The homozygotes are yet to extend their stems (bolting) and the leaf rosette appears to be less extensive. Floral development also appears to have progressed further in the azygotes as complete flowers may be seen. The homozygote is still undergoing bud formation at this point. It should be noted that both populations were of exactly the same age and had be grown in parallel under identical conditions.

Further differences were observed as development progressed and are also shown in Figure 4.5. The homozygous plants did eventually develop flowers and at an apparent higher density than the azygotes although the siliques formed did not contain seeds. This was not a result of sterility as it was possible to mechanically induce self-fertilisation. The homozygous plants seemed to be more highly branched than the azygotes and there were considerably fewer aerial leaves produced from the shoots. The final height of the homozygotes did not approach that of the azygotes; this can be seen in Figure 4.6. The plants life cycle is almost entirely complete (leaf senescence and Silique formation can clearly be seen) and still the homozygote is considerably smaller than the other plants, so showing that the reduced height was not merely the result of delayed growth.

4.4 The Production of an Anti-AtCAP1 Antibody.

It was important to confirm that the SALK 112802 insertion line shows altered AtCAP1 expression in order to implicate a lack of functional CAP as the cause of the phenotype described in Section 4.3. It was believed that the insert would either result in the expression of a truncated CAP protein or lead to the absolute elimination of expression. This was to be achieved by raising an AtCAP1 specific antibody and using it to probe a total protein extract taken from a homozygote.

Figure 4.5: The SALK 112802 Insertion has a Visible Phenotype in Mature Plants.

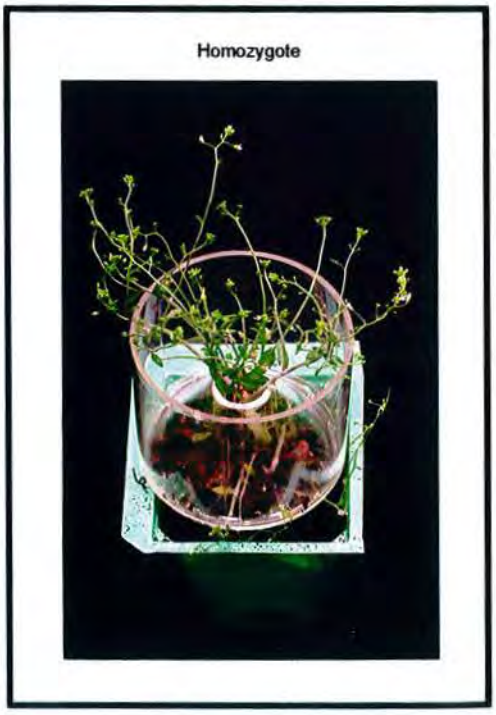


Fig 4.5: Mutant plants show delayed bolting and dwarfism (top panel). This image was taken one week after transplantation onto soil and it can be seen that the Azygotes are significantly larger. Further differences are observed as development progresses (bottom panels). The mutant stems show considerably more branching and it appears that each stem develops a greater number of flowers.

Figure 4.6: SALK 112802 Mutants Display Considerable Dwarfism.

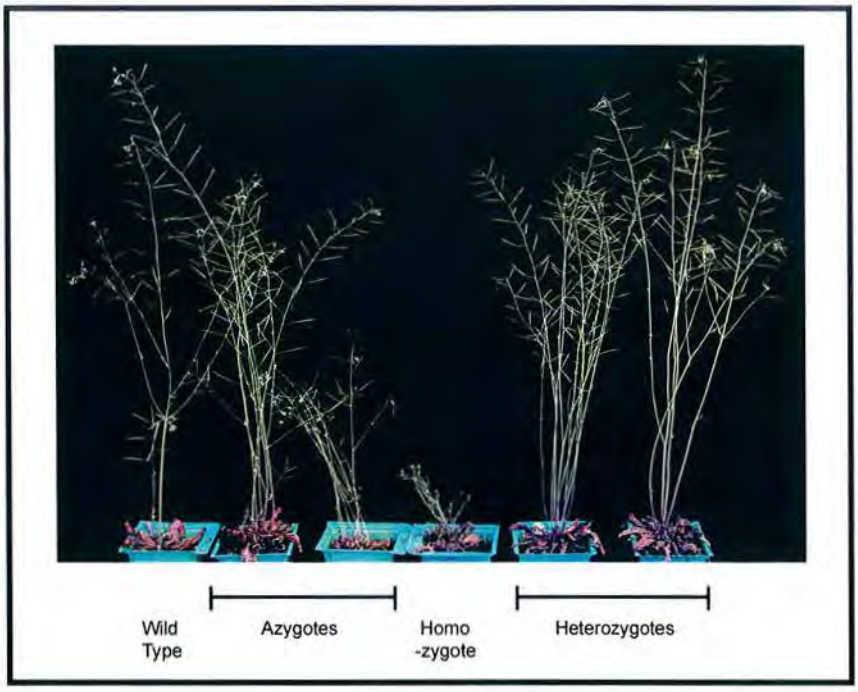


Fig 4.6: It can be seen that the Homozygote plant is significantly smaller than the others. This image was taken eight weeks after transfer to soil and leaf senescence is clearly visible. It can therefore be said that the mutants will not achieve a similar height to the Azygotes.

The availability of AtCAP1 epitopes was a prerequisite for the production of an antibody and this was achieved by cloning a fragment of the gene and expressing it in an *E.coli* host (strain BL21-DE3). The expressed fragment corresponded to the 147 N-terminal residues of AtCAP1 and also included a six Histidine tag in order to simplify purification. This length was selected in order to provide as many epitopes as was possible whilst avoiding a moderately hydrophobic stretch of the protein that may have complicated expression. The fragment was amplified via PCR reaction (see Figure 4.7a) and cloned into the pDONR-201 entry vector via a Gateway site-specific recombination reaction (see Section 2.2.1.3). The fragment (which included Gateway *att* sites, the Histidine tag, a linker sequence and 441 base pairs of AtCAP1) was then sub-cloned into the expression vector pGAT-4 (a derivative of PET28a). This vector was transformed into the expression cells and a culture of CAP-expressing cells was grown before the recombinant AtCAP1 fragment was extracted and purified (as described in Section 2.2.3.2). The eluted proteins were then examined by SDS-PAGE and an example of such a gel is shown in Figure 4.7b. It can be seen that a product of approximately 19 K Da (the expected size of the construct) is present but the majority of the protein is contained within two smaller bands.

It was thought possible that construct has been cleaved into smaller fragments in which case the samples could still be used for the production of an anti-CAP antibody as CAP epitopes would still be present. This was confirmed by probing a Western Blot of the eluted fractions with an anti-Histidine tag antibody. The blot is shown in Figure 4.7c and it can be seen that all eluted fractions contain two protein fragments that strongly cross-reacted with two protein fragments. Firstly this demonstrates that the construct was being expressed in the *E.coli* cells and secondly the presence of two separate Histidine-tagged bands indicates that cleavage had indeed occurred. It was decided that the fragmented protein would still be suitable for antibody production and so the proteins were prepared as described in Section 2.2.4.1 and subsequently given to the staff of the Life Sciences Support Unit.

4.5 Assessing the Affinity and Specificity of the anti-AtCAP1 Antibody.

Protein extracts from mature *Arabidopsis* and *E.coli* expressing a full length AtCAP1 construct (see Section 5.1) were used to test the polyclonal antibody that had been raised against the N-terminal AtCAP1 fragment. Both extracts were size fractionated on a 12% SDS-PAGE Gel and were electro-blotted onto a membrane. This was then probed with the primary polyclonal antibody diluted 1:500 and a commercially produced anti-mouse Horseradish peroxidase-conjugated secondary antibody. The results are presented in Figure 4.8 and it can be seen that the antibody has cross-reacted with a band of the correct size in both samples, although there is significantly more background in the *Arabidopsis* lane. This is a result of having to expose the image for a significant amount of time (5 minutes), which was rendered necessary by the weakness of the signal. An increase in antibody concentration (to 1:200) eliminated this problem in subsequent blots and allowed a clean band to be detected (see Figure 4.11). It is interesting to note that the difference in size between the wild-type Arabidopsis protein, with a predicted molecular weight of 52 KDa and the recombinant protein with a predicted molecular weight of 54 KDa is visible upon the gel.

4.6 The Expression of AtCAP1 within SALK 112802 Homozygous Plants.

Protein extracts were made from two azygous plants, one heterozygote. one homozygote and a wild-type plant for the purpose of comparing AtCAP1 expression levels. The concentration of these samples was measured by the process described in Section 2.2.3.4 and an equalised amount of each sample was size fractionated on a 12% SDS-PAGE gel. This was then transferred to a membrane via electro-blotting and probed with the anti-CAP antibody as previously described. The image obtained from this Western blot is shown in Figure 4.9 and is presented in conjunction with an Amido black stain of the membrane. The Amido Black stain of the membrane demonstrates that the total amount of protein present in each sample was equal. It can clearly be seen that the anti-CAP antibody has labelled a band in all of the samples with the exception of plant homozygous for the SALK 112802 insert. It is concluded that

Figure 4.7: The Cloning and Expression of an N-Terminal Fragment of AtCAP1.

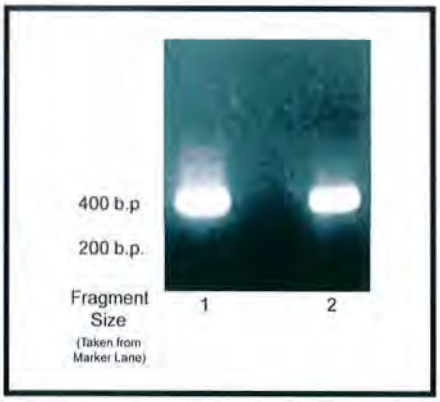


Fig 4.7a: The 440 Base Pair Fragment was amplified from an *Arabidopsis* cDNA library by PCR. Each reaction was performed in duplicate and the products were visualised following Gel Electrophoresis. A marker lane was run in an adjacent well; the product sizes were estimated by comparison to the standards.

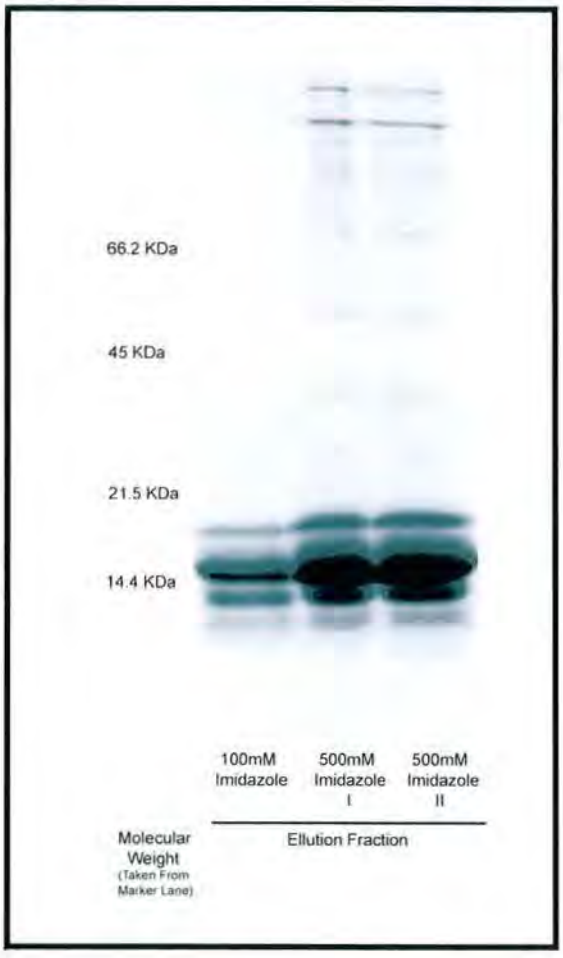


Fig 4.7b: The Fragment was subcloned into an expression vector and transformed into an *E.coli* expression system. Expression was induced via the addition of IPTG and the recombinant protein was purified on the basis of it's interaction with a Nickel affinity column and elluted with buffer containing Imidazole. The fractions were applied to a 15% Poly-acrylamide gel and seperated by SDS-PAGE. The protein bands were visualised with Coomassie stain and their molecular weight was estimated by comparison to standards in an adjacent lane.
The expressed fragment was estimated to have a molecular weight of approximately 19KDa. The most abundant proteins appear to have a molecular weight slightly in excess of 14KDa which is suggestive of protein cleavage.

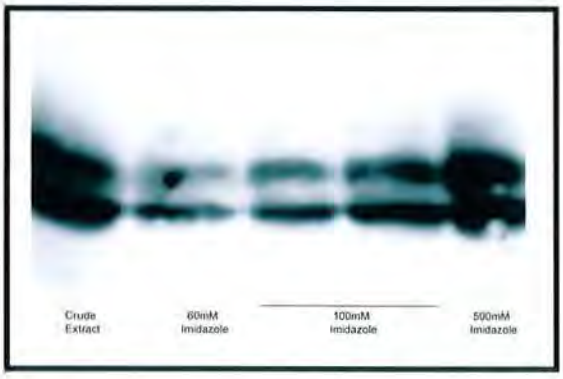


Fig 4.7c: Selected fractions were electro-blotted onto a nitrocellulose membrane and probed with an anti -His Tag antibody. The presence of the His Tag within two of the bands again suggests cleavage.

Figure 4.8: The Mouse anti-AtCAP1 Polyclonal Antibody Recognises a Single Protein Band in Extracts from Both *Arabidopsis* AtCAP1-Expressing *E.Coli.*

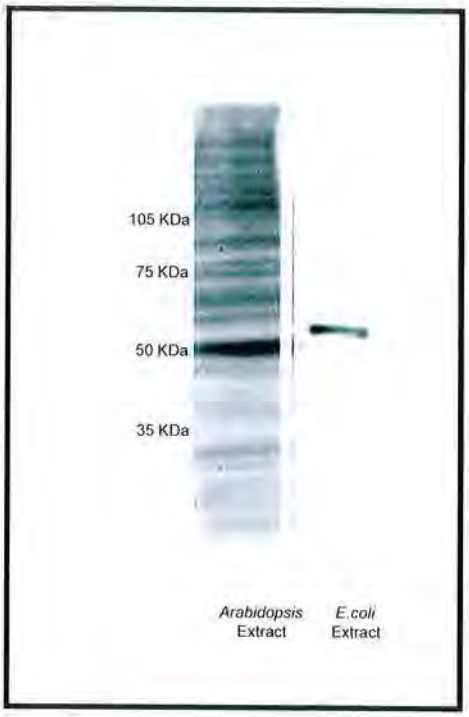


Fig 4.8: Total protein extracts were taken from both a mature *Arabidopsis* plant and a sample of the AtCAP1 *E.coli* expression culture. These were size fractionated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The blot was then probed with the anti-CAP antibody at a dillution of 200:1. It can clearly be seen that the antibody has detected a single band of the correct size (i.e. 52K Da) in both samples. It is interesting to note that the recombinant AtCAP appears to run higher on the gel. This is a result of the fused His Tag and Gateway linker sequence.

Figure 4.9: The Salk 112802 Mutant Line Lacks AtCAP1 Expression

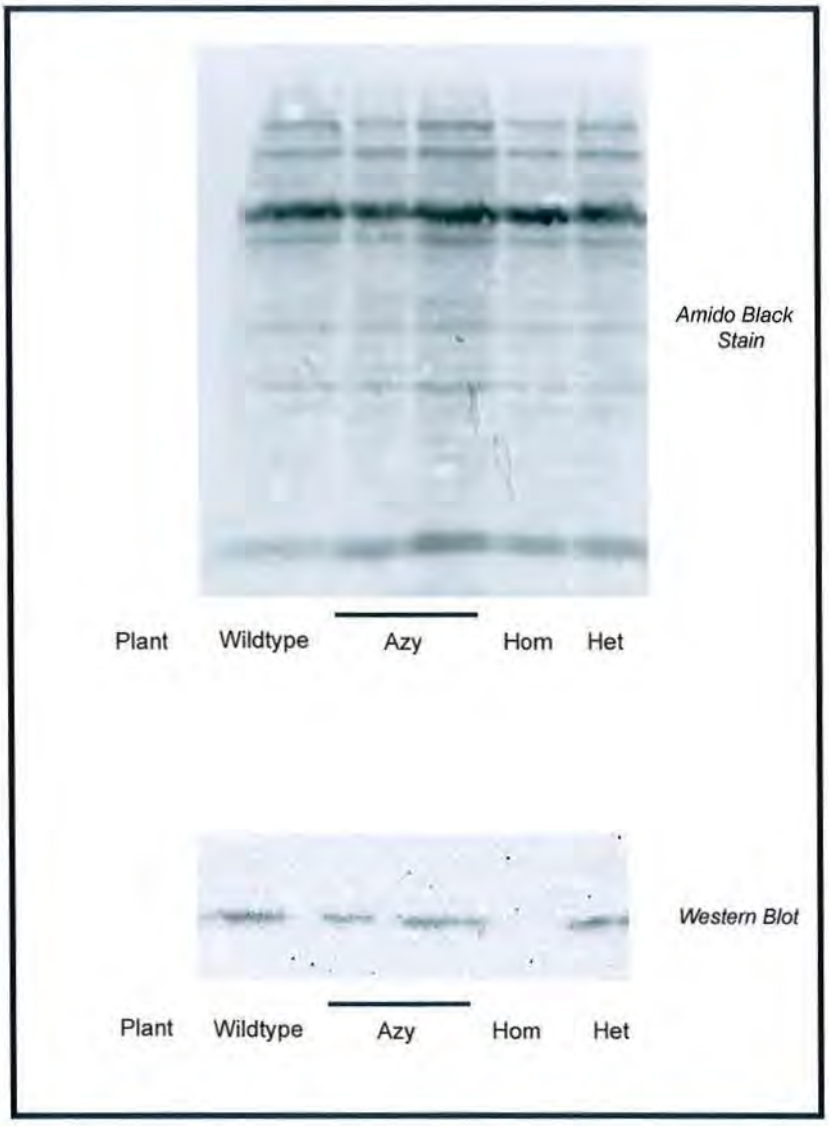


Fig 4.9: Total Protein extracts were taken from plants Azygous, Heterozygous and Homozygous for the SALK 112802 T-DNA Insert and separated on a 12% Poly-accrylamide gel by SDS-PAGE. An extract from a Wildtype plant (Columbia ecotype) was also used. The proteins were transferred to a nitrocellulose membrane by electro-blotting and probed with the anti-AtCAP1 polyclonal antibody in conjuction with a Horseradish Peroxidase -conjugated anti mouse secondary antibody. The blot was visualised and the absence of a band in the lane corresponding to the Homozygote can clearly be seen. A band is visible in the other lanes. The blot was then stained with Amido Black in an attempt to visualise all bound protein and demonstrate equal loading.

this plant did not express AtCAP1 and therefore may serve as a model for AtCAP1 knockout

4.7 The Transmission of the AtCAP1-deficient Genotype.

The transmission ratio of the mutation was measured by growing the progeny of a single heterozygous plant and individually genotyping the seedlings. In total 69 plants were successfully genotyped and the results are shown below in Table 4.4:

Table 4.4: The Transmission of the SALK 112802 Allele.

	AZYGOTES	HETEROZYGOTES	HOMOZYGOTES
Frequency	33	32	4
Percentage	47.83	46.38	5.8

In addition it was noted that plants homozygous for the insertion did not produce seed unless they were self pollinated via external intervention. The ratios obtained deviate from the expected Mendelian transmission ratio of 25:50:25% Azygote:Heterozygote:Homozygote significantly and there are a number of possible reasons for this. Firstly it is possible that the AtCAP1 deficient embryo's exhibit reduced viability and so are less likely to mature into viable seeds. This possibility was tested by examining the siliques produced by heterozygous plants as a certain number of absent or deformed embryo's would be expected were this mutation an embryo problem. Images of the dissected siliques are shown in Figure 4.10, it can be seen that all of the developing seeds are of a similar morphology and there aren't any obvious gaps left by failed embryos.

An alternate explanation for the reduced transmission would be reduction in the viability of CAP-deficient pollen. A comparison of the pollen produced by azygous and heterozygous plants is shown in Figure 4.12 and it can be seen that there are some differences in appearance between the two pollen types. The CAP deficient pollen appears to be rounder as well as having a more concave surface. The overall size of the pollen grains however is comparable.

Several attempts were made to assess the viability of the CAP-deficient

Figure 4.10: Seed Development in SALK 112802 Heterozygous Siliques

Fig 4.10: The siliques of heteozygous plants display no obvious phenotype. No underdeveloped or withered embryo's are visible and there are no gaps where development has failed to occur.

Figure 4.11 : A Morphological Comparison of WildType and CAP-Deficient Pollen

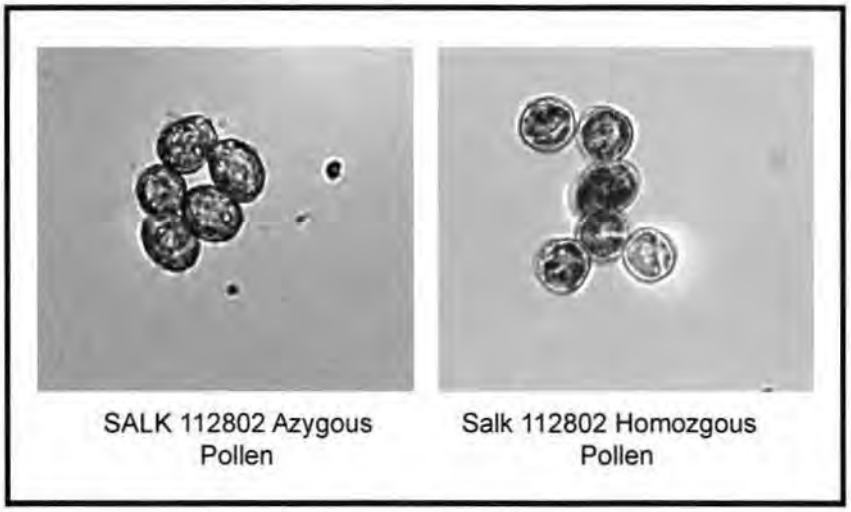


Fig 4.11: Pollen from both populations were exmained under a light microscope using a 40x long working distance objective. Both samples appear to be of a similar size and to have a similar external morpholgy.

pollen by determining its ability to germinate in artificial media. Germination rates of approximately 30% were achieved with pollen grains taken from azygous plants whereas it proved impossible to germinate any of the CAP-deficient pollen using the same protocols (as described in Section 2.2.5.1)

4.8 The Interaction of AtCAP1 with F-Actin in Pollen.

It was decided to investigate the interaction between by CAP and the Actin cytoskeleton in Pollen grains in an attempt to explain the reduction in pollen viability caused by the SALK 112802 insertion. The labelling of Actin in the pollen grains of *Narcissus* is a well-established and optimised procedure and so it was decided to use this model. In addition *Narcissus* pollen is significantly larger than that of Arabidopsis, meaning that cellular detail would be more readily observable. The cross reactivity of the anti-AtCAP1 polyclonal antibody to other plant CAP family members was assessed by probing total protein blots from tissues that may have been relevant to future work (including *Narcissus* pollen) with the antibody at a 1:200 dilution. The result of this may be seen in Figure 4.12 and the cross reactivity of the anti-AtCAP1 antibody can clearly be seen.

Fresh pollen was collected, fixed and digested before finally being stained with the anti-AtCAP1 antibody (in conjunction with a TRITC-conjugated anti-mouse secondary antibody) and Bodipy-conjugated Phallodocidin. The grains were imaged under a Laser Scanning Confocal Microscope and an example is shown in Figure 4.13. The co-localisation of the *Narcissus* CAP homologue and it's F-Actin network is apparent when the images obtained from the two channels are merged. The considerable complexity of both networks is better illustrated by the higher resolution and higher contrast monochrome images also presented in Figure 4.13.

The interaction between CAP and the Actin network in germinating pollen tubes was also of interest and so pollen tubes that had been permitted to germinate for two hours were labelled and examined in a manner similar to the pollen grains. A typical example of one of these germinating tubes is presented in Figure 4.14 and it can be seen that there are no obvious fibrous or filamentous structures visible on the CAP channel suggesting a diffuse

Figure 4.12: The Anti-AtCAP1 Polyclonal Antibody Cross-Reacts With Other Plant CAP Family Members

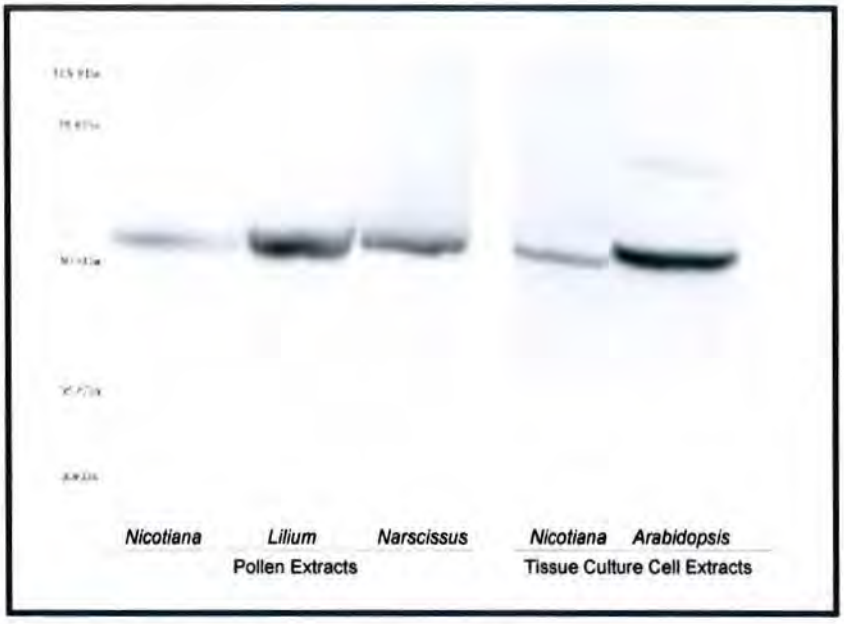


Fig 4.12: Total protein extracts from selected plant tissues were size fractionated by SDS-PAGE and Electro-blotted onto a Nitrocellulose membrane. This was then probed with the anti -AtCAP1 polyclonal antibody (diluted 1:200) and a Horseradish peroxidase-conjugated secondary antibody. It can be seen that a single protein band is clearly labelled in all of the samples although a weaker band with a higher molecular weight is visible in the Arabidopsis sample. The control molecular weights were taken from an adjacent marker lane.

Figure 4.13: CAP Co-localises with F-Actin in Pollen Grains.

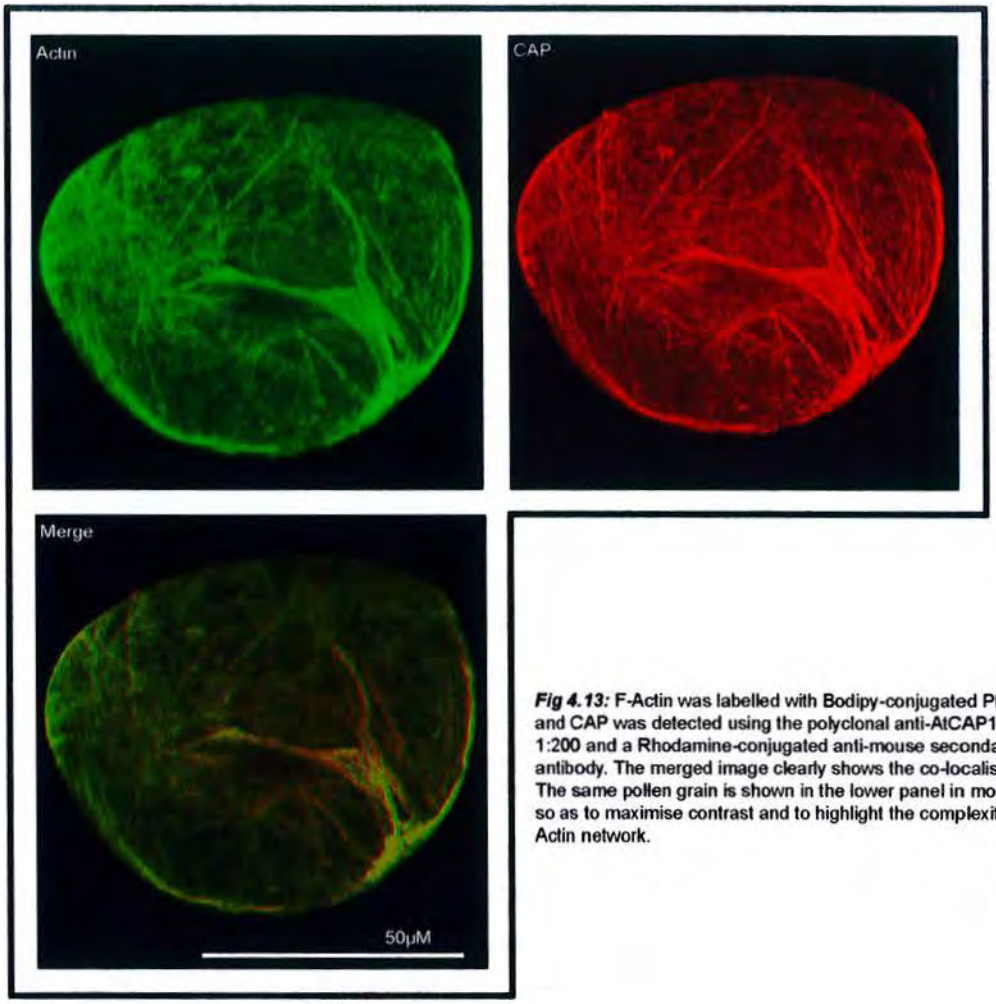


Fig 4.13: **F-Actin was labelled with Bodipy-conjugated Phalloidocadin and CAP was detected using the polyclonal anti-AtCAP1 antibody at 1:200 and a Rhodamine-conjugated anti-mouse secondary antibody. The merged image clearly shows the co-localisation. The same pollen grain is shown in the lower panel in monochrome so as to maximise contrast and to highlight the complexity of the Actin network.**

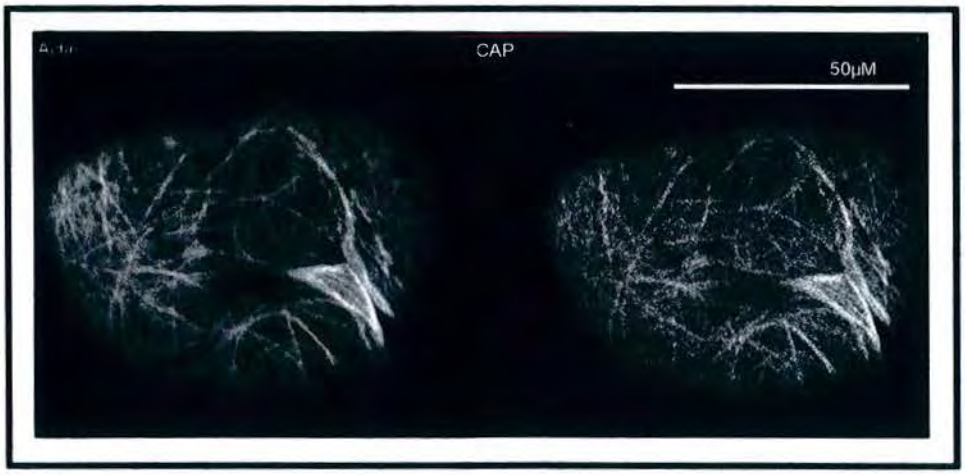


Figure 4.14: CAP Does Not Interact With F-Actin in Germinating Pollen Tubes

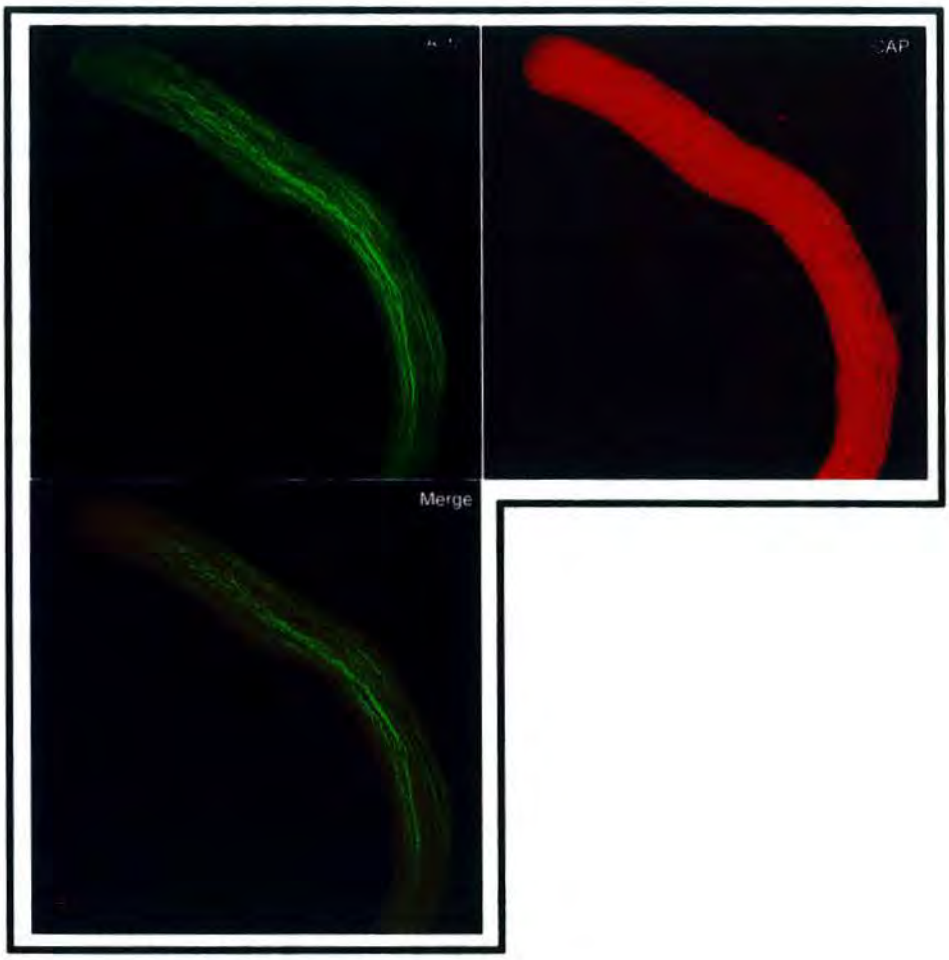
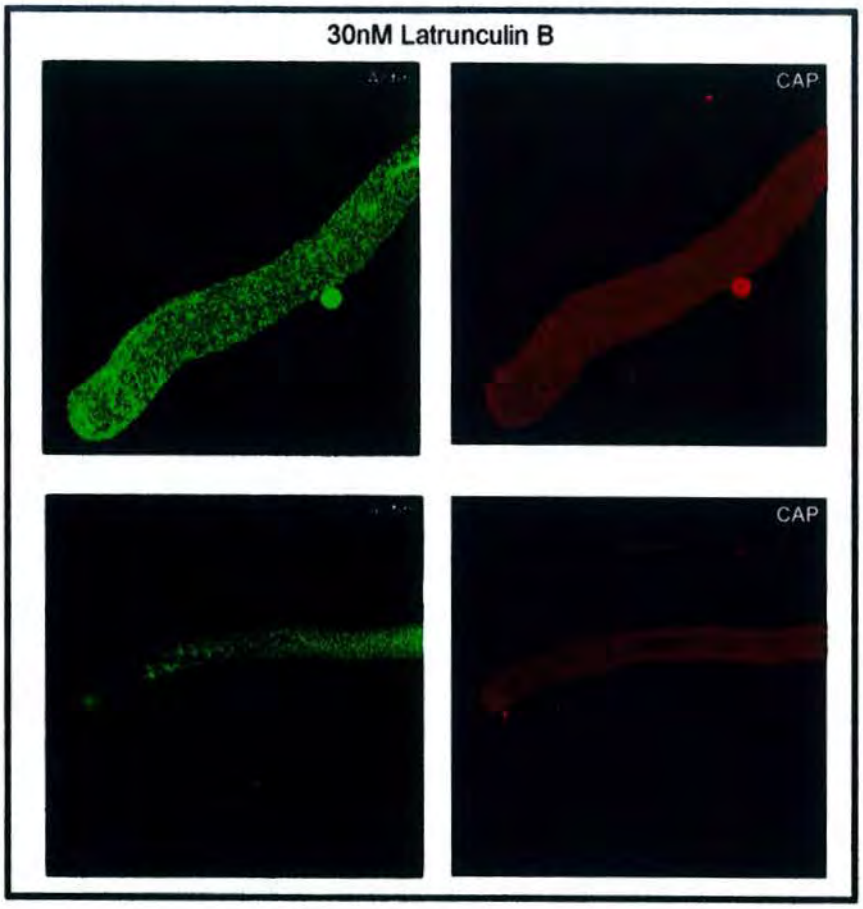


Fig 4.14: Actin was stained with Bodipy cojugated Phallodocidin and CAP was labelled with the polyclonal anti-AtCAP1 antibody dilluted 1:200 and a TRITC-conjugated anti-mouse secondary antibody. It can be seen that CAP does not co-localise with filamentous Actin and appears to be distributed throughout the cytoplasm.

Fig 4.15 AtCAP1 Does Not Interact with Drug Induced F-Actin Structures Within Pollen Tubes



cytoplasmic distribution. This is confirmed by the lack of co-localisation shown by the merged image.

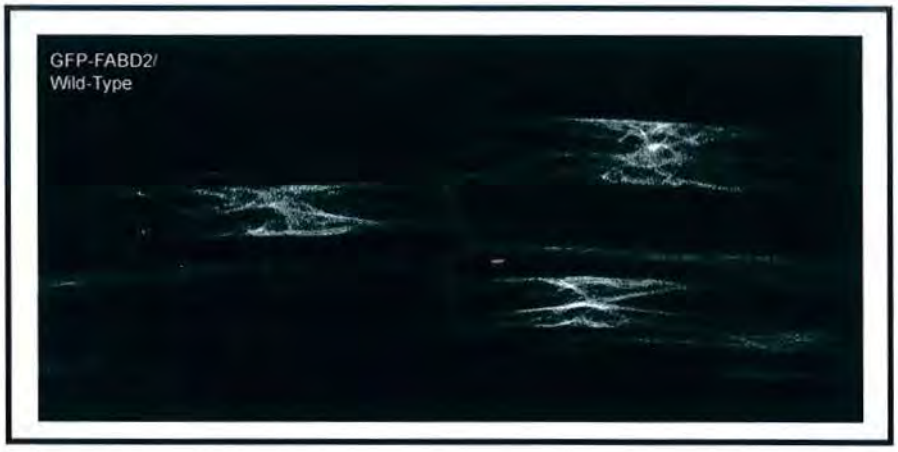
Previous work performed upon other Actin binding proteins, namely ADF (Smertenko *et al* 2001) has shown them to decorate artificially-produced actin structures such as the spicules generated by exposure to Cytochalasin D. Pollen tubes were treated with either 30 µM Cytochalasin D or 30 nM Latrunculin B for 30 minutes prior to fixation and again were stained with the anti-CAP antibody and Bodipy-conjugated Phallodocidin. Typical examples of the observed pollen tubes are presented in Figure 4.15. It can be seen that exposure to Cytochalasin D has resulted in the F-Actin network condensing into condensing in thick spikes (or spicules) of Actin which was as expected but it can also be seen that they are not decorated with CAP. Likewise incubation with Latrunculin B has resulted in the destruction of Actin filaments and the formation of dense actin aggregates to which CAP has again not bound.

4.9 The Effect of AtCAP1 Knockout upon the Actin Cytoskeleton of Somatic Cells.

The previously described phenotypes are suggestive of disruption to the cytoskeleton and so it was desired to image the network of mutant plants. This was achieved by crossing a line expressing a Green Fluorescent Protein Fimbrin Actin-Binding Domain 2 (GFP-FABD2) construct into a SALK 112802 homozygote. The GFP construct had previously been shown to be able to label the actin cytoskeleton *in vivo* without affecting its dynamics to the extent of a Talin based construct (Ketelaar e*t al* 2004b). The actual cross was performed by Dr. Michael Deeks and his assistance is acknowledged.

Progeny generated by the cross were genotyped and assessed for levels of GFP expression. Several homozygotes of the F2 generation were found to show acceptable levels of fluorescence and so were dissected and examined. Figures 4.16 and 4.17 show the effect of the elimination of AtCAP1 expression on two different types of cell, namely roots cells and shoot epidermal cells. In both cases it can be seen that that the mutants have severely disrupted actin cytoskeletons. The extensive fine actin network of the wild type root cells has been replaced by a significantly smaller array of filaments and it appears as if either the nuclear basket has thickened or excess Actin has been

Figure 4.16: The SALK 112802 Insertion Disrupts the Actin Cytoskeleton in Root Cells.



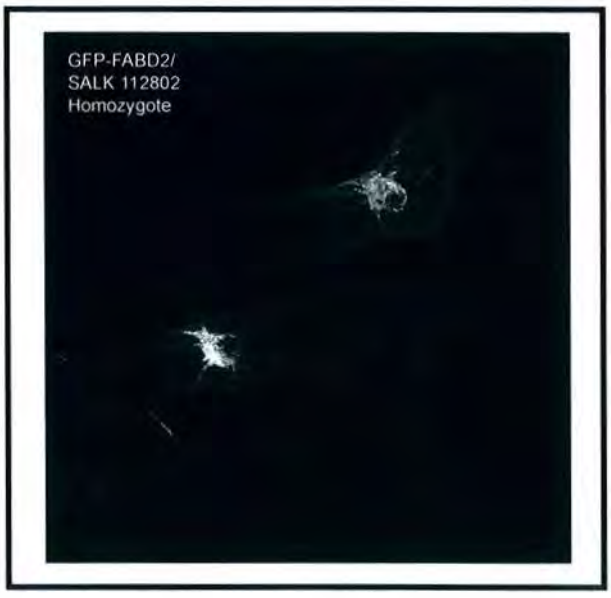


Fig 4.16: The elimination of AtCAP1 expression severely disrupts the actin cytoskeleton. It can be seen that CAP- cells have a less extinsive network of actin filaments and it appears as if a considerable amount of F-Actin has collapsed onto the surface of the Nuclear membrane.

Figure 4.17: The Loss of AtCAP1 Expression Disrupts Actin Organisation in Other Cell Types

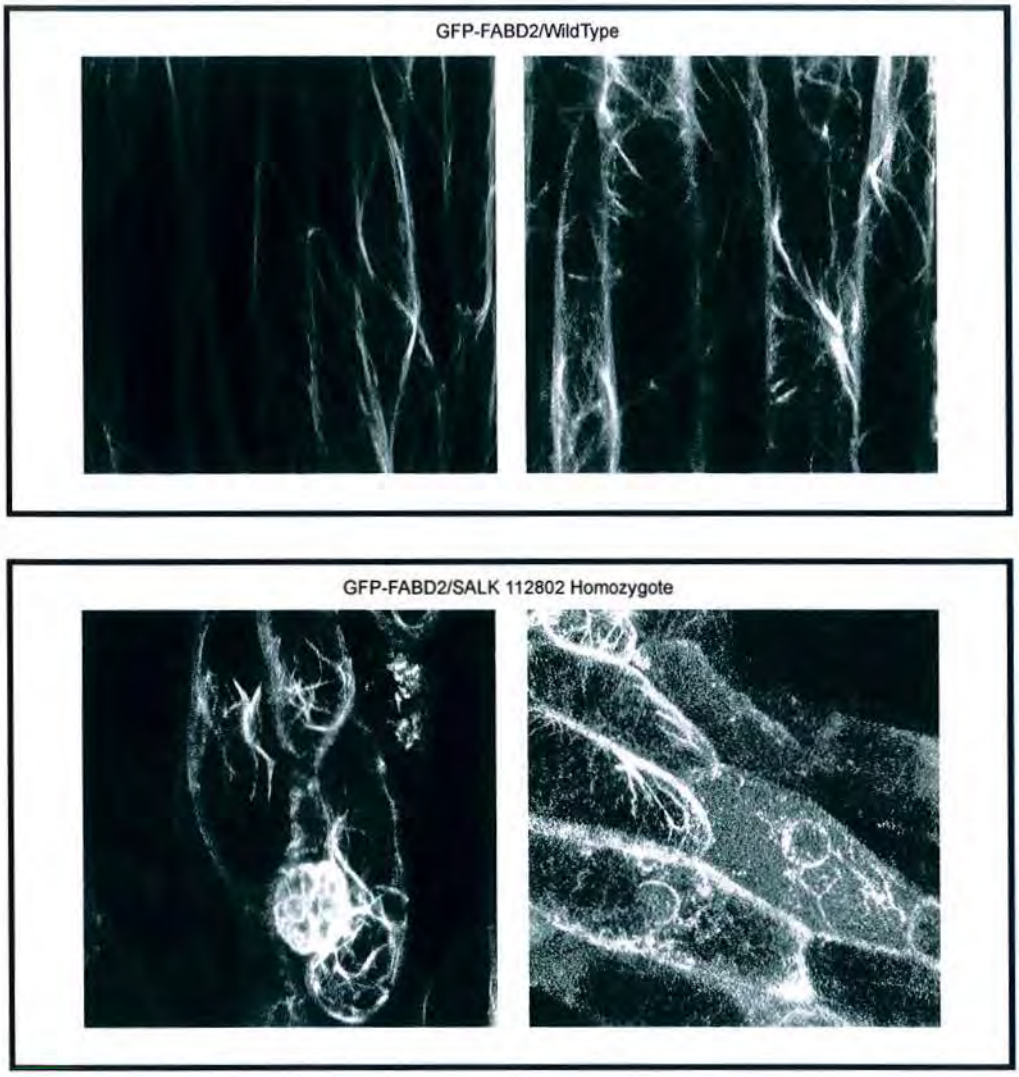


Fig 4.17: Examination of cells from the stems of both mutant and wild-type plants again highlights a significant difference in Actin organisation. The Actin network is less extensive in the mutant cells and again it appears as if significant amounts of F-Actin have become deposited upon the surface of the nuclear membrane. In additon the cells appear to be both shorter and rounder than those of the wild-type plant.

indiscriminately deposited into the nuclear region. Comparison of the wild type and mutant shoot cells shows that the elongate fine actin networks of wild type cells have been replaced by short and dense irregular filaments in the mutant. In addition it appears as if aggregates of actin are present in the cytoplasm and again the nuclei appear to have gained a substantial coat of actin. The mutant shoot cells also appear to have taken on a different shape in that they are considerably shorter that those of the wild type and appear to be more bulbous. The examination of these cells under bright-field illumination (Figure 4.19) confirms this observation and also reveals the mutant cells to have a somewhat irregular shape and arrangement in comparison to the long and regular files of the wild type.

4.10 The Regulation of Root Hair Development by Cyclic AMP Modulating Drugs.

The absence of the root collar is a phenotype that has not previously been described as occurring in cytoskeletal mutants and so it was hypothesised that it may be a result of a disruption of cAMP signalling. The role of cAMP in root hair development was investigated by vernalising root hairs in the presence of 40μM Forskolin (an activator of Adenylate cyclases) and 100μM SQ 22,536 ([9-(tetrahydro-2-furanyl)-9-H-purin-6-amine, a potent inhibitor of Adenlyate cyclase activity). Forskolin has previously been shown to mediate an affect upon pollen tubes at this concentration (Moutinho et al 2001) and SQ22, 536 has been shown to completely inhibit hormone induced cAMP production in Guina-Pigs via inhibition of the adenylate cyclase (Turcato and Clapp 1999). The seeds were then transferred to media containing the same compounds at the same concentrations and measurements pertaining to root and root hair development were taken. These included the overall length of the primary root, the length of root hairs and the density of initiation. The datas collected are presented below in Tables 4.5 to 4.7 and are show graphically in Figure 4.17.

Figure 4.18: Cells from the Stem of SALk 112802 Homozygotes are Irregularly Shaped and Do Not Fully Elongate

Fig 4.18: These brightfield images show that cells in the mutant plants have an irregular shape and are significantly smaller than those from the wild -type plant (both samples were taken from the same region of the stem). It apppears that the mutant cells have not elongated to the same extent as the wild types

Table 4.5: The Effect of cAMP-Modulating Drugs Upon Root Length.

Treatment	Mean Root Length (mm)			Standard Deviation Of 6 Day Sample (mm)
	2 Days	4 Days	6 Days	
Wild Type	1.88	9.21	21.79	3.91
40 μM Forskolin	1.45	8.36	20.67	5.29
100 μM SQ22,536	1.76	6.67	17.72	4.58

The differences between the samples are less that the Standard Deviation values associated with each population as there was great diversity in the length.

The stated time of measurement was the number of days post germination.

Table 4.6: The Effect of cAMP-Modulating Drugs Upon Root Hair Length.

Treatment	Mean Root Hair Length (mm).	Standard Devaition (mm).	Standard Devaition as a % of Mean Length (%).
Wild Type	0.31	0.18	59.3
40 μM Forskolin	0.33	0.22	68.2
100 μM SQ22, 536	0.42	0.24	57.9

Again it can be seen that the difference in mean root hair length between the samples is significantly less than the Standard Deviation of each sample thereby rendering the small observed difference invalid.

Table 4.7: The Effect of cAMP-Modulating Drugs Upon Root Hair Density.

Treatment	Mean Number of Root Hairs per 3mm Segment	Standard Deviation	Standard Deviation as a % of Density	Number of Root Hairs mm^{-1}	Percentage Difference from Wt (%)
Wild Type	34.67	3.64	10.49	11.56	0
40 μM Forskolin	30.00	3.38	11.27	10	-13.5
100 μM SQ22, 536	31.83	9.39	29.49	10.61	-8.2

The density of root hairs is similar to the other measured characteristics in that modulation of the plants cyclic AMP levels did not result in a significant effect. Again it can be seen that the small observed variation was, in the case of the inhibitor SQ22, 536, less than the standard deviation of the wild type sample and was only slightly in excess of this when exogenous Forskolin was added.

Figure 4.19: The Additon of Exogenous cAMP Modulating Drugs Does Not Significantly Affect Root Hair Development

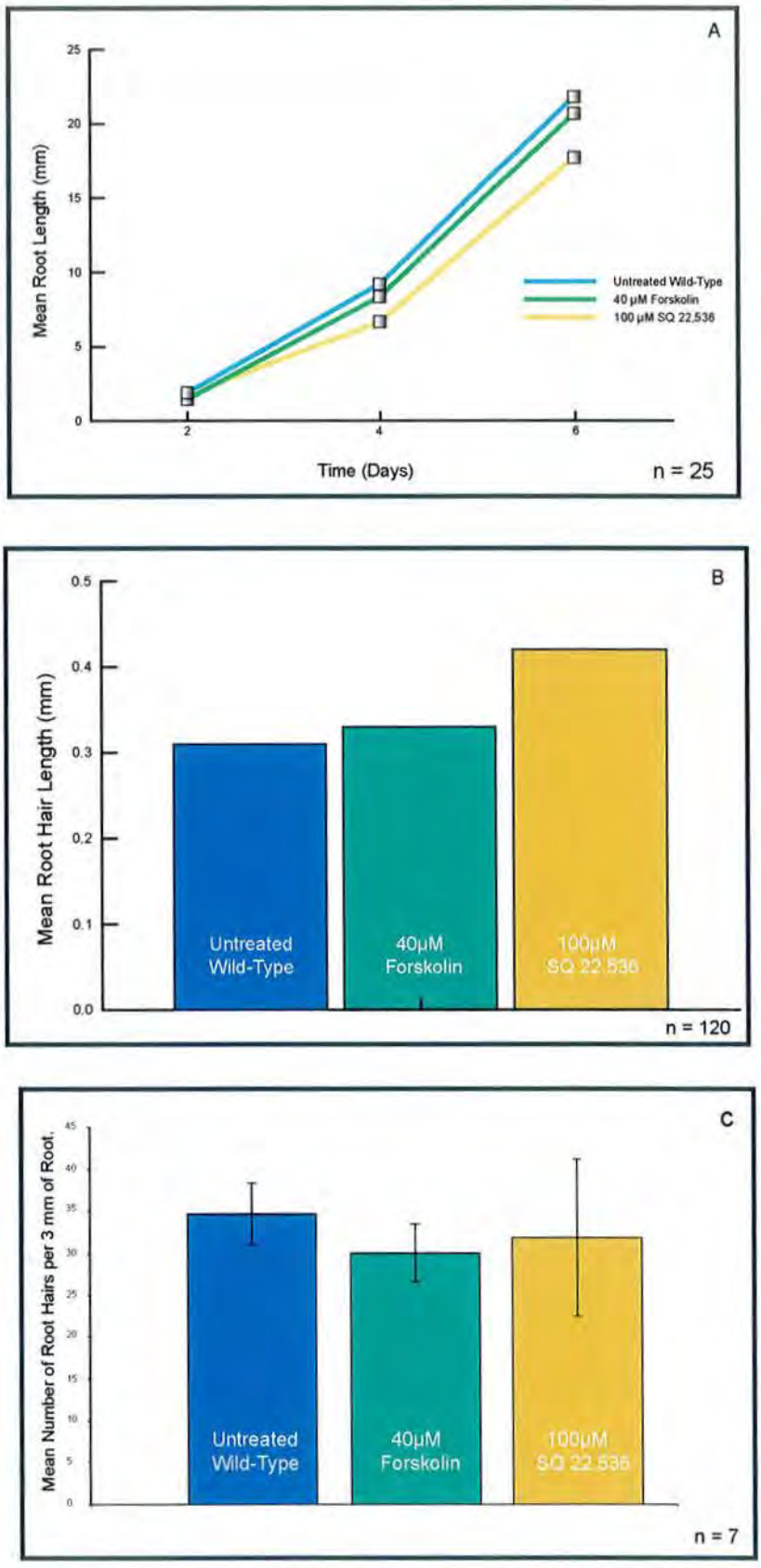


Fig 4.19: Neither the addtion of the Adenylate Cyclase activating drug Forskolin nor the inhibitor SQ 22,536 had a significant effect upon the development of *Arabidopsis* root hairs. The variation in root length (A) between the treatments was less than the samples Standard Deviation and so may be considered to be insignificant. A similar result was obtained when the mean root hair length (B) was measured. In both cases Error Bars are not shown for the sake of figure clarity. The variation in root hair density was also found to be less than the standard deviation of the sample size (C).

These experiments indicate that the root hair phenotype is not a result of disrupted cAMP signalling although confirmation that the drugs had affected cAMP levels would be of use in this experiment.

4.11 Chapter Summary

The results presented in this chapter demonstrate that disruption of the AtCAP1 locus results in a significant and distinctive phenotype. This includes the absence of an initial collar of root hairs, a reduction in root hair initiation and a dramatic reduction in root hair extension. These phenotypes are not believed to be a product of disrupted cAMP signalling and so it is proposed that a defective Actin cytoskeleton is responsible.

Later stages of plant development are also affected by an elimination of AtCAP1 expression. Overall height is significantly reduced and the aerial architecture of the plant is altered resulting in increased stem branching. The viability of the pollen also appears to be reduced although embryo development does superficially appear to be affected. Examination of CAP localisation within *Narcissus* pollen grains revealed it to strongly associate with filamentous Actin although it does not appear to be coupled with to cytoskeleton in the germinating pollen tube.

The elimination of AtCAP1 expression in Arabidopsis was shown to severely disrupt the Actin cytoskeleton in a number of cell types. This disruption resulted in a significant reduction in the size and complexity of Actin filaments and it appears as if excess Actin is deposited onto the surface of the nucleus and into cytoplasmic aggregates.

Chapter V: Interactions with the Cytoskeleton.

The main objectives of the work described in this Chapter were to:

I *Study the biochemical Interaction between AtCAP1 and Actin*

II *Investigate the architectural changes induced upon Actin by association with AtCAP1*

III *Assess AtCAP1's ability to form multimers*

IV *Use Yeast Two Hybrid technology to investigate AtCAP1's interactions with other Actin binding proteins.*

5.1 Introduction.

Interactions between proteins are critical in determining not only their localisation but also their functional role. It would be expected that a protein such as AtCAP1, which is believed to be involved in regulation of the actin cytoskeleton, would interact with other cytoskeletal components. The work presented in this chapter represents an attempt to investigate AtCAP1's ability to interact with some of the cytoskeletal proteins (including Actin) that other CAP family members have been shown to associate with. It was hoped that this would allow AtCAP1 function to be compared to some of the better characterised CAP family members and this would allow a cellular level model of Higher Plant CAP function to be devised.

These aims were to be achieved by firstly characterising the interaction of AtCAP1 with actin using biochemical methods and secondly by using Yeast Two Hybrid technology to map the interactions between AtCAP1 and other Actin interacting proteins.

5.2 The Cloning and Expression of AtCAP1.

A biochemical characterisation of AtCAP1 would require a large amount of the purified protein. For convenience was achieved by expressing AtCAP1 as a recombinant protein in a bacterial host with a Histidine tag to aid purification. The full-length gene was cloned by amplifying the coding sequence from an Arabidopsis cDNA library via a PCR reaction (see Figure 5.1a) and inserting the product into the pDONR-201 entry vector via a Gateway BP reaction. Recovered clones were then digested with the Bgl II restriction endonuclease which was predicted to release a fragment of approximately 800 base pairs from AtCAP1. The products of this digest were then run an agarose gel and the result of this is shown in Figure 5.1b. It can be seen that two of the lanes (i.e. 2D and 2E) released a fragment of the correct size and contained a linearised backbone. These clones were then sequenced using multiple primers to ensure that entire coding sequence was present and was free of mutations. The result was a useable full-length AtCAP1 clone.

The coding of sequence of AtCAP1 was then sub-cloned into the expression vector pGAT-4 (a derivative of PET28a) via a Gateway LR reaction. The presence of AtCAP1 in the expression vector was again confirmed by digestion and sequencing. This expression clone was then transformed into BL21-DE3 strain *E.coli* and a culture was established. Expression was induced by the addition of 1mM IPTG and the protein was purified on the basis of it's Histidine tags affinity with a Nickel Ion column. Considerable time and effort was spent optimising the expression protocol with factors such as the length and temperature of the expression step being varied until maximal expression was achieved. Selected highlights of this process are shown in Appendix III. The eluted fraction were analysed on a 12% SDS PAGE gel and an example of one of these is shown in Figure 5.1c. It can be seen that a good quantity of relatively pure AtCAP1 could be produced using this method. Finally, the recombinant protein was dialysed into a buffer appropriate for whichever assay it was to be used in; this would most often be G-Buffer or PBS.

Figure 5.1: The Cloning and Expression of AtCAP1

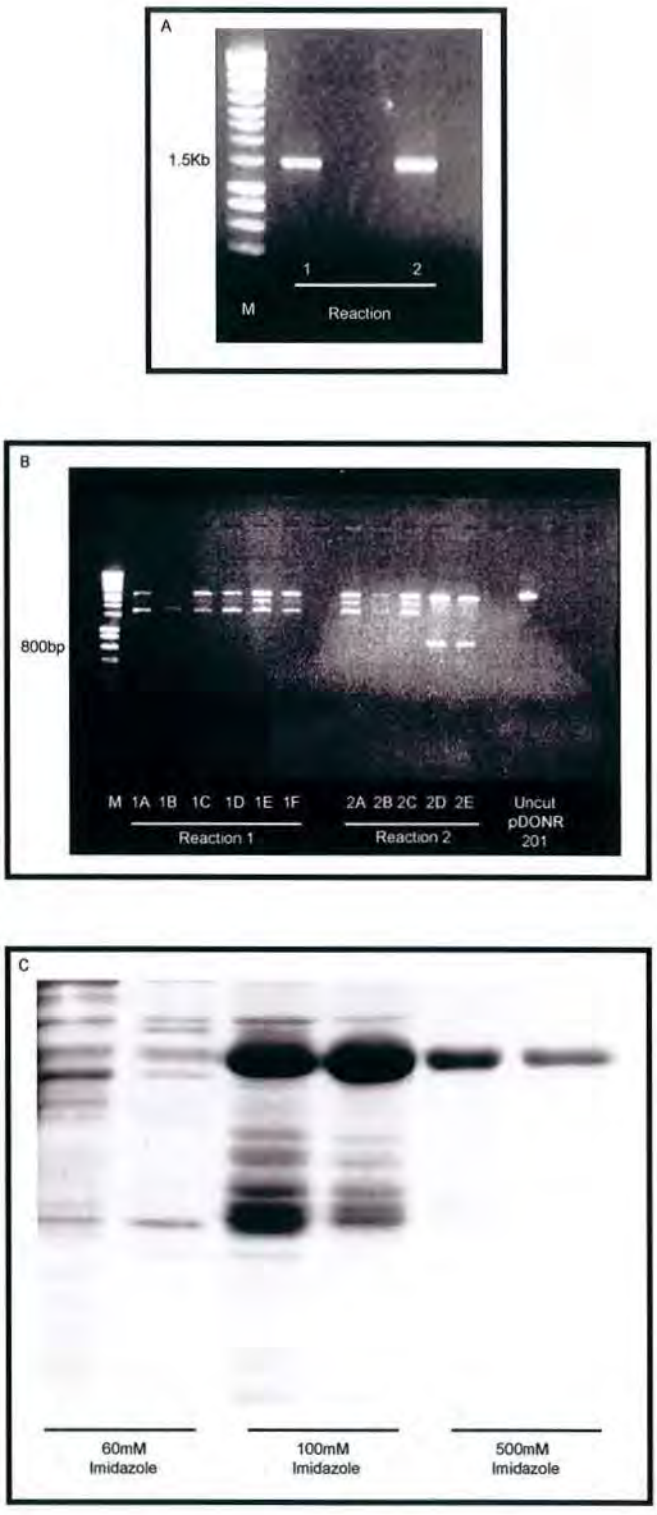


Fig 5.1: AtCAP1 was amplified from an Arabidopsis cDNA library via PCR (A) and was inserted into the entry vector pDONR-201 via a Gateway BP Recombination vector. Recovered recombination products were digested with the restriction endonuclease Bgl-II (B); it was expected that such a digest would result in the release of a 800 base pair fragment from AtCAP1 and that this would be easily detectable upon the gel. it can be seen that clones 2D and 2E potentially inculde AtCAP1. This was confirmed by sequencing. AtCAP1 was then sub-cloned into the Histidine-tagged bacterial expression vector pGAT-4 and the protein was expressed in an *E.coli* host (C).The figure shows a 12% SDS-PAGE gel of the fractions elluted by an increasing concentration of Imidazole from a Nickel affinity collumn.

5.3 The Interaction of AtCAP1 with Filamentous Actin.

Other members of the CAP family have previously been shown to be able to inhibit the polymerisation of Actin by sequestering the monomer when they are present at a sufficiently high concentration (Zelicof *et al* 1996). This is believed to show that CAP family members are able to directly interact with G-Actin. It was believed that the addition of AtCAP1 to a F-Actin co-sedimentation assay would reveal whether or not it was also able to sequester the monomer, as it would be expected that significantly less actin would enter the pellet were that the case.

AtCAP1 and commercially produced Rabbit muscle Actin were mixed at a concentration of 5 μM before a high salt KME buffer was added; this was intended to induce Actin polymerisation. The reaction was allowed to proceed for one hour at room temperature. The polymerised and un-polymerised Actin were separated by an Ultra-centrifugation step and the contents of both the pellet and the supernatant were analysed on an SDS PAGE gel. An example of this is shown in Figure 5.2.

It can be seen that the vast majority of Actin enters the pellet in both the absence and presence of equimolar AtCAP1 and so it cannot be said that AtCAP1 inhibits polymerisation even in at such a high concentration. The inability of actin to enter the pellet in the absence of KME buffer demonstrates that it's decrease in solubility was a product of polymerisation rather than non-specific precipitation or aggregation. The most surprising and interesting result was the tendency of AtCAP1 to enter the pellet in the presence of filamentous actin. It can be seen that only 12.3% of the AtCAP1 present pellets out in the absence of F-Actin but this increases to 53.8% when F-Actin is also present. This is strongly indicative of an ability to bind F-Actin.

5.4 The Effect of AtCAP1 upon Actin Filaments.

Potentially the binding of AtCAP1 to F-Actin could induce changes in the filament architecture and it was thought this possibility would be best investigated by direct visualisation of AtCAP1-bound Actin filaments. This was

Figure 5.2: AtCAP1 Binds Filamentous Actin *in vitro*.

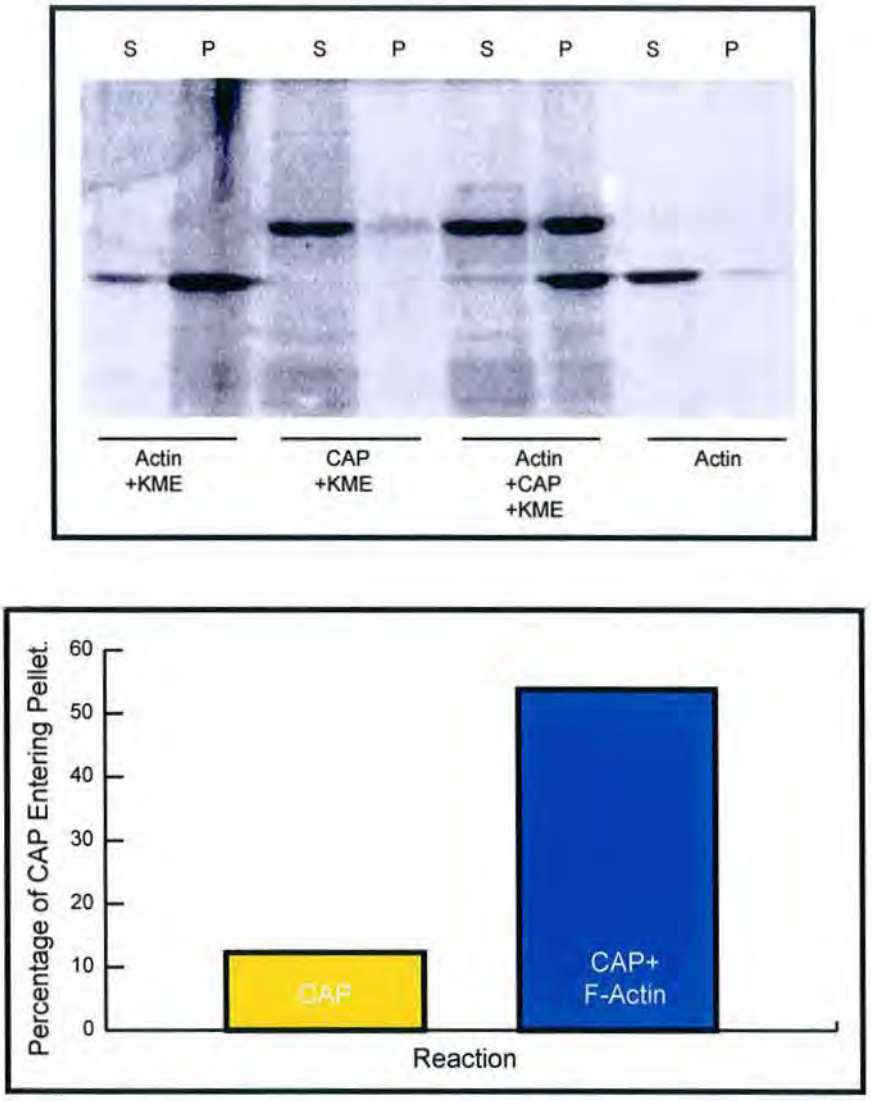


Fig 5.2: The top panel shows a co-sedimentation assay including expressed AtCAP1 and Rabbit skeletal muscle Actin. The proteins were both dilluted to 5μM in 90 μL of G-Buffer before 10 μL of 10x KME buffer was added. The reactions were incubated for one hour at room temperature. The insoluble fraction was then pelleted out by 15 minutes of centrigugation at 200,000g. Finally the supernatant and pellet from each reaction were seperated and ran on a gel in adjacent lanes (marked S and P respectively). It can be seen that CAP in isolation remains soluble but will enter the pellet when F-actin is also present. This is strongly suggestive of F-actin binding. In addition it can be seen that the pelleting of actin is dependant upon the presence of KME buffer, so showing that actual polymerisation (as opposed to precipitation or colagulation) is occuring.
The lower panel shows the amount of CAP that was found in the pellet as a percentage of the total CAP present in both fractions. This was obtained by scanning the gel and measuring the size of the band present and multiplying this value by the average (inverted) pixel brightness value.

achieved by polymerising 5 μM Actin in both the presence and absence of AtCAP1 (as before) and then transferring aliquots of the reaction to carbon-coated electron microscopy grids. The samples were then negatively stained with a 1% Uranyl acetate solution and imaged at 130,000x magnification under a Transmission Electron Microscope (TEM). The TEM was operated by Mrs Christine Richardson during this experiment and her help is very gratefully acknowledged.

Examples of the images obtained are presented in Figure 5.3 and it can be seen that the presence of AtCAP1 appears to induce the bundling of Actin filaments. Careful examination of the images suggests that each CAP induced bundle contains between four and six individual filaments although this is little more than an estimate due to insufficient image resolution. This bundling activity was seen whenever the concentration of AtCAP1 exceeded 3 μM, which would suggest that each AtCAP1 molecule was required to be in contact with multiple Actin monomers (the initial concentration of Actin was 5 μM).

5.5 The Multimerisation of AtCAP1

Attempts to investigate the multimerisation of AtCAP1 with chemical cross-linking proved to be unsuccessful (a summary of these experiments is given in Appendix IV) and so an alternate method was used. Both full-length AtCAP1 and an N-terminal fragment (441 base pairs) were sub-cloned into the Yeast Two Hybrid vectors pAS2-1GW and pAct2-1 GW which include the GAL4 transcription factors Binding Domain and Activating Domain respectively. A full overview of the Yeast Two Hybrid system is given in Section 2.2.6 and in Figures 2.3 and 2.4. These vectors were then transformed into *Saccharomyces* and cells expressing the two AtCAP1 constructs were mated with each other. Diploid cells (i.e. those that include both vectors) were selected on the basis of their ability to grow on media lacking both Leucine and Tryptophan; these are abilities conferred by genes encoded within the Yeast Two Hybrid vectors.

The diploid cells were then transferred to media lacking either Adenine and/or Histidine; both of these nutritional deficiencies may be overcome by the formation of a complete GAL4 transcription factor and the subsequent expression of the ADE2 and HIS3 proteins. The formation of the transcription factor is dependant upon the binding of the two proteins to which its binding and

Figure 5.3: AtCAP1 Induces the Formation of F-Actin Bundles.

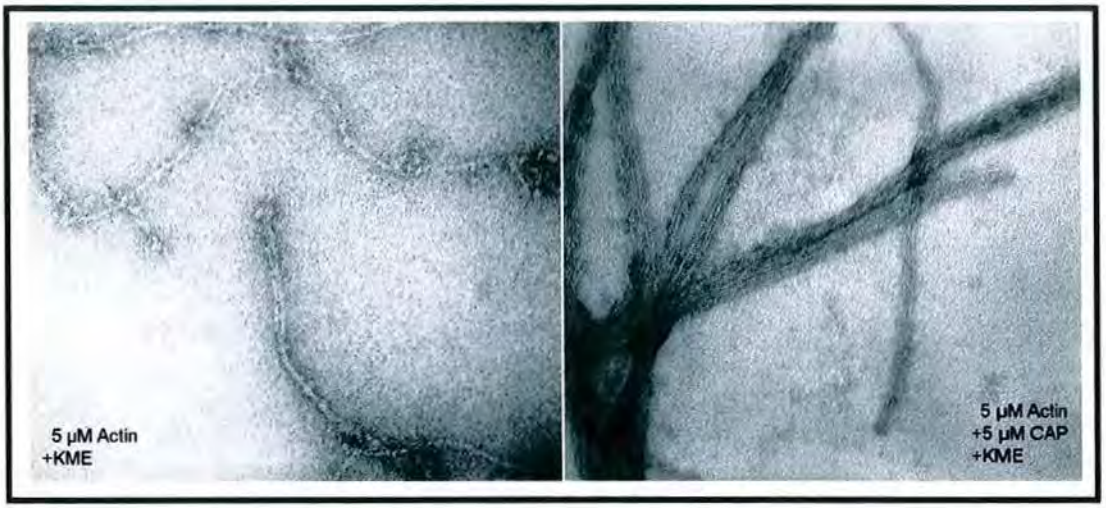


Fig 5.3: **The images show Actin filaments (stained with 1% Uranyl acetate) magnified 130,000x under a Transmission Electron Microscope (TEM). It can be seen that the Actin appears to have formed bundles in the presence of CAP. The help of Mrs Christine Richardson in operating the TEM during this experiment is gratefully acknowledged**

activating domains are fused. Therefore the survival and growth of the yeast upon the selective media are dependant upon the interaction of the two proteins being tested. The Binding Domain constructs had previously been tested to selective media to demonstrate that they weren't able to independently activate transcription of the reporter genes. Such auto-activation is a common source of false positives in Yeast two Hybrid experiments.

Figure 5.4 shows the result of two separate interaction tests, the panel to the left shows a stringent assay where both Histidine and Adenine deficiencies were imposed and the panel to the right shows the result of a more permissive assay where only Histidine synthesis was required for survival. The more stringent assay clearly shows that the N-terminal fragment of AtCAP1 was able to associate with itself and with the full-length protein when the fragment was in the binding domain. It would however appear that the same was not true when AtCAP1 was in the Binding Domain and the fragment was in the Activating Domain. In addition it would appear that the full-length clone was not able to associate with itself.

A reduction in the stringency still permitted the fragment to self-associate and to bind the full length clone (when in the Binding Domain) and additionally the full length clone was also able to self associate. It was still not possible for AtCAP1 to bind its N-terminal fragment when the full-length clone was in the Binding Domain. The Human Lamin C protein in the Binding domain served as a negative control. This clone was selected as a control on the basis of its low ability to bind other proteins; it is a commonly used Yeast Two Hybrid control.

5.6 Interactions Between AtCAP1 and Other Cytoskeletal Components.

The Yeast Two Hybrid Method was also used to investigate the interaction between AtCAP1 and other components of the Actin Cytoskeleton. The result of these experiments is shown in Figure 5.5. The top panel shows interactions between AtCAP1 and Human Cytoplasmic Actin B, *Arabidopsis* ADF2, *Arabidopsis* AIP 1-1, *Arabidopsis* Profilin 2 and *Arabidopsis* Profilin 3 with AtCAP1 being in the Binding Domain. The bottom panel shows the same interactions with the orientations reversed. Actin was re-cloned into the Gateway system from a previous vector and sub-cloned into the Yeast Two Hybrid system whereas ADF2 was cloned from an *Arabidopsis* cDNA library

Figure 5.4: Yeast-Two-Hybrid Experiments to Show the Multimerisation of AtCAP1

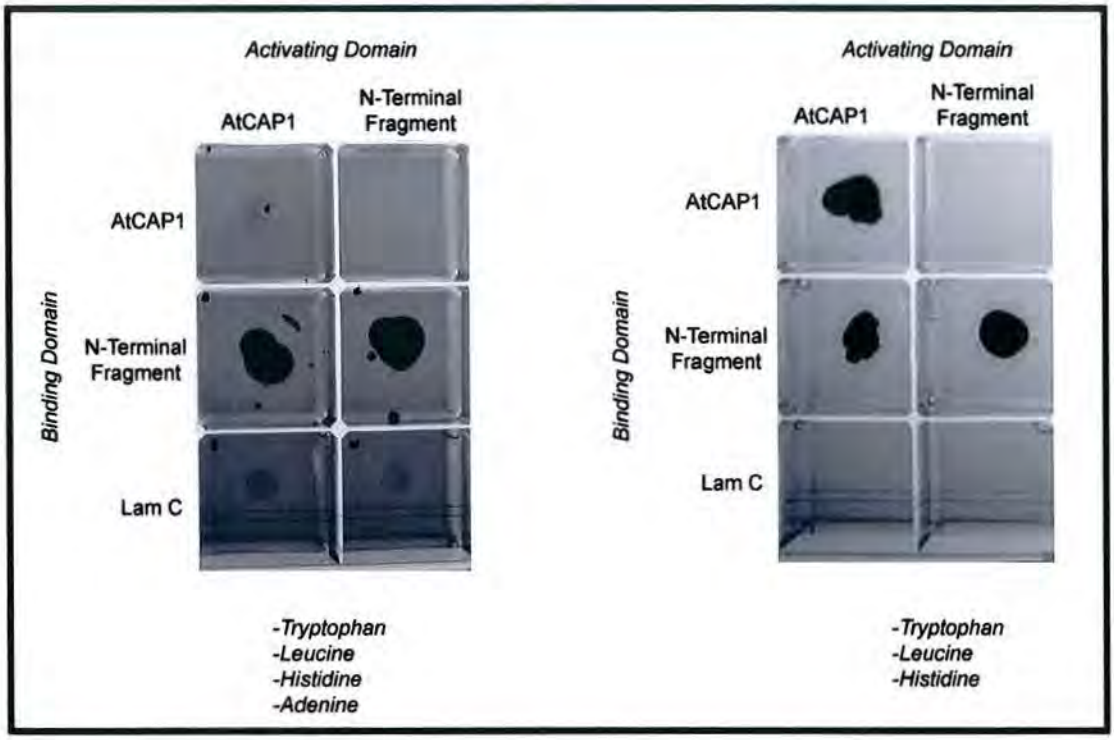


Fig 5.4: The high-stringency Yeast-Two-Hybrid interaction test (on the left) shows that the N-terminal fragment of AtCAP1 is able to interact with both itself and the full-length protein. Neither protein showed strong interaction with Human Lamin C, a coommonly used negative control. A less stringent assay (only the expression of *HIS3* is being tested) is shown to the right. This test showed a similar result to the full assay but in addition it is suggested that full-length AtCAP1 may self-associate. Yeast expressing each gene in a fusion construct with the GAL4 fragment was permitted to mate for 48 hours before diploids were selected on the basis of their ability to survive of media lacking both Leucine and Tryptophan. Surviving colonies were then transplanted onto selective media.

Figure 5.5: Interactions Between AtCAP1 and Other Components of the Actin Cytoskeleton

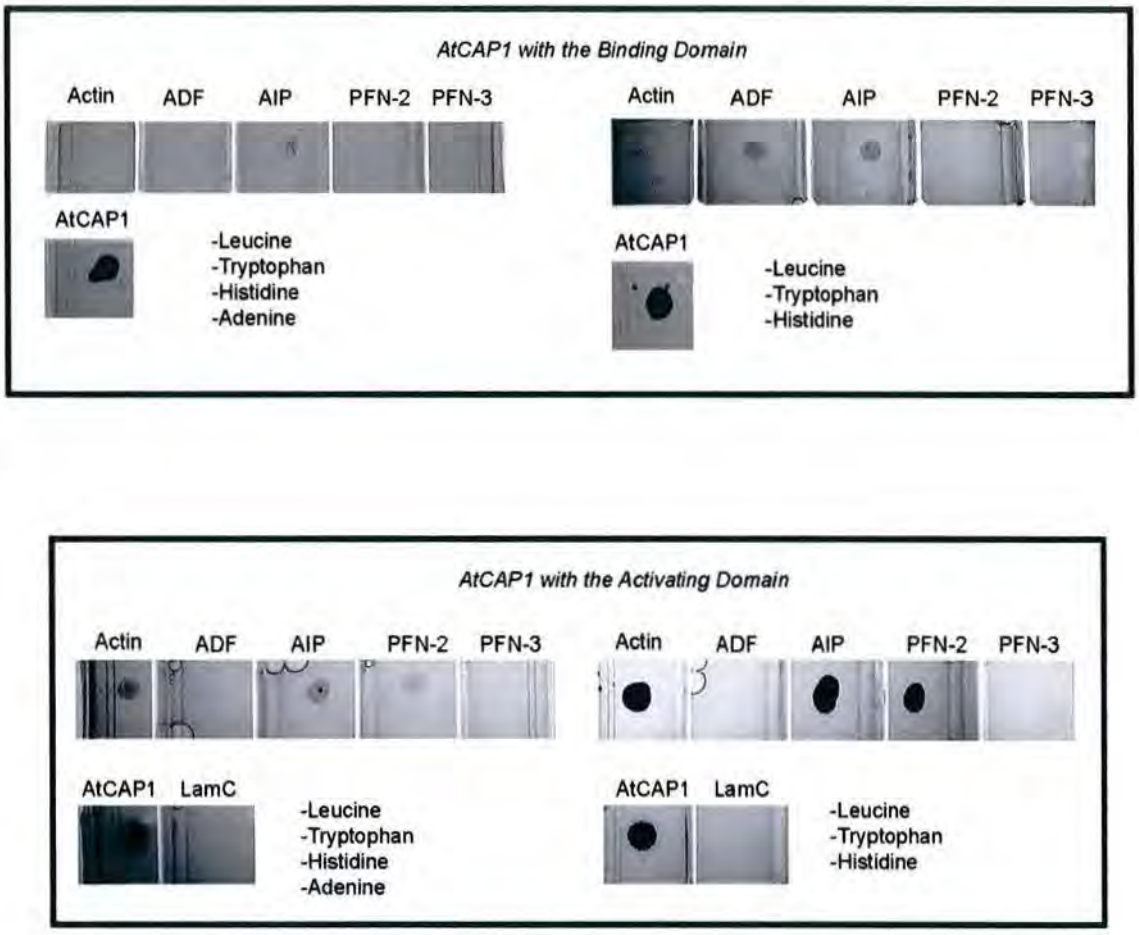


Fig 5.5: The top panel represents the result of interaction tests where AtCAP1 was conjugated to the Binding Domain of the *GAL4* Transcription factor and various other Actin binding proteins (i.e Human Cytoplasmic Actin B, *Arabidopsis* ADF-2, *Arabidopsis* AIP1-1 and *Arabidopsis* Profilins 2 and 3) were conjugated with the Activating Domain. All constructs were permitted to mate for 48 hours before diploid cells were selected (on the basis of their ability to synthesise Leucine and Tryptophan) and tested for interactions. The left of the figure shows a stringest test with growth requiring the synthesis of both Histidine and Adenine whereas the right of the figure shows a less stringent test where interactions were demonstrated solely by the ability to overcome Histidine starvation.
The lower panel shows the same interactions with but with AtCAP1 conjugated to the binding domain with the other proteins this time being conjugated to the Binding Domain of the *GAL4* transcription factor.In additon it can be seen that matings between AtCAP1 expressing yeast and the LamC negative control strain were not viable under selection.

Figure 5.6: The PFN2 and ADF2 Yeast-Two-Hybrid Clones are Able to Interact with Actin

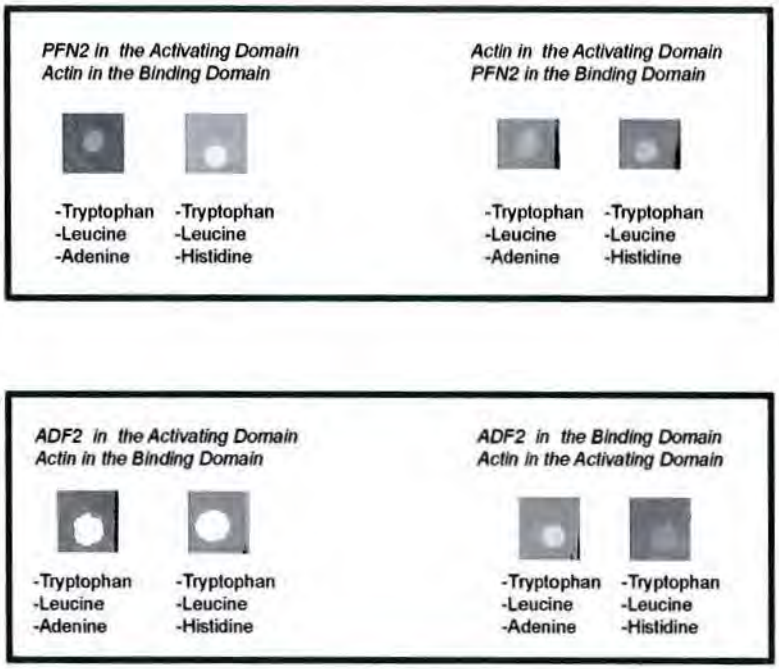


Fig 5.6: It can be seen that the PFN2 and ADF2 clones are functional as they are able to interact with Actin in both the Binding and Activating domains and on both selective media. Some interactions are stronger than others but some growth has occured in all tests.

and then sub-cloned. The AIP Yeast Two Hybrid clone was a gift from Dr Ellen Allwood and PFN's 2 and 3 were a gift from Dr Michael Deeks. Human Lamin C in the Binding Domain again acts as a negative control.

It can be seen that, in this experiment, AtCAP1 was generally a stronger interactor when in the Activating Domain. The higher stringency test shows a strong interaction with itself and reasonably strong interactions with AIP and Actin. Some interaction with Profilin 2 may also be seen. A reduction in stringency resulted in extremely strong interactions with Actin, AIP, Profilin 2 and itself although there was still no suggestion that AtCAP1 was able to bind ADF or PFN-3. There was no growth observed in the Lamin C control experiments.

Experiments with AtCAP1 in the Binding Domain yielded generally weaker interactions but it can still be seen to bind with itself in the higher stringency test and there is a possibility of a very weak interaction with AIP. A reduction in stringency results in the strengthening of the interaction with AIP and evidence of binding with ADF. There is however still no support for binding with ADF, Profilin 2 and or Profilin 3.

The inability of Profilin 3 to interact with CAP in any of the experiments and the general poor performance of the ADF clone led to questions regarding their functionality. This was investigated by testing their ability to interact with the actin clone and the result of these test matings is shown in Figure 5.6. It can be seen that Profilin 3 was able to overcome both nutritional sensitivities in both interaction orientations and shows good growth in all tests. This is suggestive of a strong interaction. ADF2 showed extremely good growth in both tests when in the Activating domain but growth in the Histidine deficiency test was weaker when in the Binding Domain. In general, though the ADF clone appeared able to bind with Actin and so was almost certainly functional.

5.7 Chapter Summary

The results presented in this chapter strongly suggest that recombinant AtCAP1 is able to bind with filamentous Actin, but does not inhibit the polymerisation of Actin filaments. In addition, it appears as if the recombinant AtCAP1 protein organises Actin filaments into regular ordered bundles. The results of the Yeast Two Hybrid experiments clearly show that AtCAP1 is able

to self-associate and that the N-terminal section of the protein is involved in this activity. It is unclear to what extent such multimerisation occurs.

Further Yeast Two Hybrid interaction experiments strongly suggest that recombinant AtCAP1 is able to associate with AtAIP1-1, AtProfilin 2 and G-Actin. These experiments gave no indication that AtCAP1 was able to bind AtProfilin 3 and the results pertaining to AtADF2 were unclear.

Chapter VI: Discussion

6.1 Introduction

The results presented in the preceding chapters demonstrated the result of eliminating AtCAP1 expression upon the growth and development of a higher plant. In addition, the effect of this disruption upon the cellular Actin network was shown and the interaction between AtCAP1 and assorted components of the cytoskeleton was demonstrated. The nature of the interaction between AtCAP1 and Actin itself was also investigated. There was no evidence to suggest that these phenotypes were a result of disruption of cyclic AMP signalling.

This Chapter discusses these results within the wider context of the plant Actin cytoskeleton and its role in development. Further experiments that may clarify some of the described finding are suggested in conjunction with discussion of those findings and suggestions for significant future work are presented in Section 5.5. Comparisons between AtCAP1 and other members of the CAP family are made and this leads to a higher plant specific model of CAP function being proposed.

6.2 The Developmental Role of AtCAP1

6.2.1 The SALK 112802 Insertion

The absolute segregation of the observed phenotype with the SALK112802 homozygous phenotype was observed over three generations during the course of this study. This continued segregation and the lack of observed phenotype within the Azygotes and Heterozygotes precludes the possibility that the phenotype is a production of additional tDNA insertions. This coupled with the absolute pentrance of the phenotype, the confirmation of the insertions position and the discovery that AtCAP1 expression was eliminated in homozygotes renders it likely that the observed phenotype is a result of a lack of active CAP. The occurrence of a phenotype is unsurprising as it has been

shown that AtCAP1 is, unlike the Profilins or ADF's, a single copy gene and it shows limited homology to other Actin associated proteins. Therefore, there is little scope for redundancy in the system

There is however a remote possibility that the insertion of the tDNA into the second exon of AtCAP1 has resulted in the disruption of neighbouring genes. Such interference could be caused by either the de-stabilisation of their mRNA or the disruption of essential promoter sequences. Examination of the genomic sequence of *Arabidopsis thaliana* Chromosome IV reveals AtCAP1's closet neighbours to be a Glycosyl Hydrolase (At4g34480) and a predicted Serine/Threonine Protein Kinase (At4g34500). It is unlikely that the elimination of a Glycosyl Hydrolase (a biosynthetic enzyme) would result in the phenotype associated with the SALK 112802 insertion but the kinases functional role is entirely uncharacterised meaning that its targets cannot be predicted. The subsequent (unpublished) discovery of a similar phenotype in an alternative CAP insertion line (GABI-KAT453G08) by Mike Deeks reduces the probability of the SALK 112802 phenotype being caused by additional tDNA insertions. Alternatively, elimination of the phenotype by complementation with a wild-type AtCAP1 allele would also remove any doubt regarding the origin of the phenotype.

Work towards the complementation of the phenotype has already been performed, to date the genomic sequence of AtCAP1 and it's promoter have been successfully cloned and sub cloned into a plant expression vector. The sequence of AtCAP1's exons has been checked to confirm a lack of mutations and the vector has been transformed into *Agrobacterium*. The transfer of this vector to SALK 112802 line plants has not, as of yet, been successful.

6.2.2 The Role of AtCAP1 in Root Development.

The SALK 112802 mutants showed a dramatic root hair phenotype that included the absence of an initial collar of root hairs, significantly reduced root hair extension and a moderate decrease in root hair initiation. The elongation of root hairs is dependant upon the delivery of additional cell membrane material to the growing tip. This is achieved by the fusion of exocytotic vesicles with the cell membrane at the apex and is dependent upon Actin-guided vesicle-trafficking (see Section 1.3.3). The presence of a fine sub-apical Actin network

is believed to confine the site of vesicle fusion to the growing tip (Ringli *et al* 2002, reviewed by Hussey *et al* 2003) and so promote focussed unidirectional extension.

It is proposed that the inability of CAP-deficient root hairs to fully extend is a result of a disrupted Actin network that in turn is a consequence of the absence of correct filament organisation. It is possible that such disruption prevents the delivery of Golgi-derived vesicles to the growing apex (due to a failure of Actin-dependant vesicle trafficking) so preventing the supply of membrane components to the tip. The role of CAP function in Actin dynamics was discussed in Section 1.5.2. Alternatively it can be seen that multiple cell types within the SALK 112802 mutant show a significant reduction in their fine Actin network (see Section 4.9) and it has previously been shown that the destruction of the sub-apical fine actin network prevents root hair elongation (Miller *et al* 1999) despite broad (vesicle-guiding) actin bundles still being present. The importance of the sub-apical fine Actin network to tip growth is further stressed by the discovery that plants expressing a dominant-negative form of the GTPase ROP2 possess a reduced sub-apical Actin network (Mathur *et al* 1999) and show reduced hair growth (Jones *et al* 2002). It is therefore not unreasonable to suggest that it is the loss of fine Actin in the CAP mutants that it is the origin of reduced root hair extension. This in turn implies that AtCAP1 plays an important role in organising the sub-apical fine Actin network and in doing so aids the guidance of vesicle fusion. It is interesting to note that the elimination of CAP expression in *Drosophila* cells also resulted in the incorrect organisation of fine Actin at the cell cortex (Baum *et al* 2000).

A reduction in the expression of an Arabidopsis AIP1 protein (another Actin binding protein, which was discussed in Section 1.2.5.3) has been found to result in a decrease in root hair growth and has also been shown to result in the loss of fine F-Actin (Ketelaar *et al* 2004). Conversely, the elimination of ADF expression has been shown to have the opposite effect; the root hairs of ADF-under-expression mutants are significantly longer than the wild type (Dong et al 2001) and feature increased amounts of longitudinal Actin filaments. This is paradoxical as ADF and AIP are believed to act synergistically to promote Actin dynamics (Ono 2001) and both are believed to associate with members of the CAP family (Matilla *et al* 2004).

It is proposed that the sub-apical fine Actin network is the product of a complex interplay between multiple Actin binding proteins, some of which are yet to be discovered and that the correct filament architecture is a product of many proteins influencing Actin dynamics. The activities of the various Actin modulating proteins are finely balanced by a sophisticated regulatory mechanism and disrupting the activity of one component will unbalance the system. The elimination of AtCAP1 expression removes one element of this regulatory system and results in an inability to correctly arrange the Actin network. It has been proposed that CAP family members and the proteins that they associate with serve to accelerate Actin dynamics by promoting the re-cycling of monomers (Balcer *et al* 2003). It could therefore be suggested that the elimination of AtCAP1 expression reduces the amount of available polymerisable monomer and therefore the cells ability to generate additional filaments. Support for this proposal is provided by the observations that monomer turnover was significantly reduced in CAP-deficient human cells (Bertling *et al* 2004). The specific role of AtCAP1 upon Actin dynamics will be discussed more fully in Section 5.4.

An examination of the organisation of Actin within the root hairs of SALK 112802 homozygotes would be a useful experiment to perform. It is believed that such an experiment would confirm the absence of fine Actin (as was observed within other cell types) and so lend support to the hypothesis that AtCAP1 is essential for Actin organisation and cell growth. The examination of mutant root hairs was attempted during this study but proved unsuccessful for technical reasons. The SALK 112802/GFP-FABP2 line has been donated to another laboratory in the hope that this examination may be conducted.

The elimination of AtCAP1 expression led to a reduction of the observed root hair density (see Table 4.3), which is potentially of interest given the uncertainty regarding the role of the cytoskeleton in root hair initiation. The application of cytoskeleton depolymerising drugs has been shown not to affect the formation of root epidermal cell bulges (see Section 1.3.3) and therefore it was concluded that root-hair initiation was a cytoskeleton independent process. This view was contradicted by the discovery that knockout of the *Act2* Actin gene leads to irregular bulge positioning (Ringli *et al* 2002) and that the expression of a constitutively active ROP2 mutant increases the overall density of root hair formation (Jones *et al* 2002). It has been proposed that Actin is

involved in promoting the expansion of the root hair and confining growth to its tip once the cell has already become committed to developing a root hair (Hussey *et al* 2003). It is suggested that the SALK 112802s apparent reduction in root hair density is a result of a failure of the actin network to promote the development of an epidermal cell bulge to a small root hair. These undeveloped bulges were of insufficient size to be visible under the objective lens used for the measurements and therefore were not counted.

A thorough search of the literature has revealed no other mutations that result in the absence of the initial root hair collar. This would appear to be a characteristic unique to CAP deficient mutants. There is a paucity of information regarding the *Arabidopsis* root hair collar and the mechanics of its development are entirely unknown. The application of the cAMP modulating drugs Forskolin (a potent membrane-soluble activator of Adenylate cyclases) and SQ22, 536 (an inhibitor of cAMP production) to wild-type plants failed to mimic the phenotype. Both drugs have previously been shown (Moutinho *et al* 2001 and Turcato and Clapp 1998) to induce an *in vivo* effect at the concentrations used in this experiment. It was not possible to directly measure the levels of cAMP within the developing plants and therefore it has to be assumed that levels of cAMP were indeed altered but it would appear as if the disruption to root hair development is not a result of disrupted cAMP signalling. The levels of cAMP within the tissue could be measured using the method of Ichikawa *et al* (1997), in essence the cAMP is removed from the tissue by passing an extract through a commercially available affinity column and eluted in an assay buffer. The amount of cAMP present is then quantified using a commercial ELISA kit. A similar procedure could be used to compare the amount of cAMP present in the CAP-deficient mutants to that found in comparable azygotes.

6.2.3 The Role of AtCAP1 in Above Ground Growth.

Plants homozygous for the SALK 112802 mutation were found to exhibit several above-ground phenotypes; these included a reduction in overall height, an increase in the amount of stem branching and an increase in the density of flowers (see Section 4.5). Wild type *Arabidopsis* (and many other plants) usually undergo a process known as bolting where the stem rapidly elongates as a result of both mitotic division in the shoot meristem and cell elongation. In

many species this is a response to long days and results in the elevation of the floral tissues (Salisbury and Ross 1992).

Elongation of newly divided shoot cells occurs by Intercalary growth (see Section 1.3.5 and results in the formation of files of extended cells (as shown in Figure 4.18). It is believed that this form of growth is primarily driven by an increase in cellular turgor pressure forcing an increase in the cell volume. The development of thick microtubule bands perpendicular to the cells long axis results in the deposition of parallel cellulose microfibrils. These are believed to oppose radial expansion and so ensure that the direction of expansion parallels the cells long axis (Mathur and Hulskamp 2002). The involvement of the Actin cytoskeleton in this process is not entirely understood but the occurrence of a fine Actin network at the point of cell expansion has been noted (Fu *et al* 2002) and the process is inhibited by Actin depolymerising drugs (Baluska *et al* 2000).

It is proposed that the fine Actin network at the site of expansion plays a role similar to the fine sub-apical Actin network in growing root-hair cells in that it guides and confines the fusion of membrane containing vesicles to the correct site. The absence of AtCAP from SALK 112802 homozygotes results in the disorganisation and reduction of this fine Actin network and therefore the addition of supplementary membrane is compromised. It is interesting to note that the elimination of AIP1 also resulted in reduced cellular extension and the loss of fine Actin (Ketelaar *et al* 2004) given that this knockout also had a similar phenotype to the CAP mutant in root hairs. It would appear that the co-operation of AtCAP1 and AtAIP1 in generating fine Actin networks is conserved amongst *Arabidopsis* tissues. It would be of interest to study the effect of double mutations upon the organisation of Actin within these cells and the impact upon cell expansion and plant growth. In particular, it would be interesting to see if an AtCAP1/AtAIP1 double mutant showed a more severe phenotype than shown by either in isolation. An AtCAP1/ADF double mutant would also be of interest; do CAP and ADF have an antagonistic effect upon cell expansion *in vivo* as has been observed for AIP and ADF in root hairs (reviewed by Hussey *et al* 2003)?

There is little evidence to support the *direct* involvement of either cAMP signalling or the Actin cytoskeleton in flower development so the apparent increase in floral density in SALK 112802 homozygotes is superficially puzzling. Floral development does require the occurrence of basic Actin-dependant processes such as mitotic division but the survival of the CAP-deficient mutants

suggests that basic cellular functions are not significantly inhibited. In essence floral development begins with the recruitment of four cells towards the edge of the floral meristem that are then believed to be influenced by locally produced Auxin and subsequently undergo differentiation into flower primordia (Kieffer and Davies 2004). It is believed that the PIN1 auxin efflux transporter is critical for the transport of auxin into these flowers as the *pin1* mutants are commonly found to lack floral primordia; a phenotype that can be rescued by the addition of exogenous auxin to the region (Reinhard *et al* 2000). It has been demonstrated that the Actin network plays an important role in regulating the positioning of PIN family members (Friml *et al* 2002) and therefore the direction of auxin import. Examination of the CAP mutant however shows that developed flowers do form and so it is not believed that the absence of AtCAP1 is influencing floral development in this way. It instead proposed that the aforementioned disruption of Intercalary growth results in reduced separation between primordia-forming groups of cells. This results in the development of a similar number of flowers to the wild type but within a smaller space so giving a higher flower density. A similar explanation would account for the apparent increased 'bushiness' of the mutants as spacing between sections of the stem predetermined to form branches would also be decreased as a result of the inhibition of Intercalary growth

6.2.4 The Role of AtCAP1 in Pollen Development.

The mutant allele of the SALK 112802 line shows reduced transmission, which could potentially be indicative of several different types of developmental problem. It is possible that the absence of AtCAP1 adversely effects embryo development and so reduces the viability of homozygous embryos with the result being that fewer of them progress to mature viable seeds. Examination of SALK 112802 Siliques (see Figure 4.10) suggests that this is not the case as all of the positions appear to contain a healthy developing embryo as opposed to a withered or aborted example. The seeds produced by the heterozygous plants were found to show a similar level of germination efficiency to the progeny of the azygotes and commercially produced wild type seeds. This confirms that the absence of CAP-deficient homozygotes was not a result of a reduced ability to germinate.

A failure in gamete development could also result in a reduction of transmission; the up-regulation of AtCAP1 expression in developing pollen (see Section 3.4) raises the likelihood of a male-specific problem. It was found that pollen from CAP-deficient plants had a different surface morphology and appeared to be less viable (measured as a lower germination efficiency). A series of reciprocal crosses between AtCAP1 mutants and wild-type plants would confirm that the reduced transmission is due to a male-specific problem. It would be expected that the transfer of wild type pollen onto the stigma of mutant plants result in the formation of entirely heterozygous progeny but the transfer of homozygous pollen onto a wild type stigma would result in reduced transmission of the mutant allele.

It is beyond the scope of this thesis to describe the formation of pollen in excessive detail but in essence the formation of pollen grains begins when a microspore mother cell undergoes a meiotic division to produce haploid cells. These will then become polarised (the nucleus will migrate to one pole) and undergo an asymmetric division that will result in the formation of a vegetative cell and a smaller generative cell. The generative cell will then be enveloped within the cytoplasm of the vegetative cell before (in *Arabidopsis*) undergoing a further division; this results in the formation of two sperm cells. Finally the grain will dehydrate and dehisce from the anther. The vegetative cell will germinate and form a pollen tube upon contact with a compatible Stigma; this will convey the sperm cells to the female gametes.

An exhaustive study into the development of *Nicotiana tabacum* pollen has highlighted the important role played by the Actin cytoskeleton in regulating the process (Zonia 2000). It was found that some of the later stages of microspore development were severely inhibited by the application of the Actin disrupting drugs Cytochalasin D and Latrunculin B. Both of the drugs prevented the retention of the nucleus at the tetrad cells pole prior to the asymmetric division (which was also disrupted). This resulted in mis-positioning of the generative cell within its vegetative host and an alteration in the nuclear morphology of subsequent developmental stages. It was hypothesised by the authors that the retention of the nucleus at one of the microspore poles by the Actin network was the origin of polarity prior to the asymmetric division. There is an interesting parallel between the dependency of the pollen cell upon the Actin network in establishing polarity and the similar role played by Actin in retaining

mRNA's at the poles of *Drosophila* cells. The elimination of Drosophila CAP expression was found to disrupt the Actin network to the extent that the mRNAs were no longer retained and polarity was not established (Baum *et al* 2000).

It is unclear to what extent the absence of AtCAP1 disrupts the development of pollen as the grains are still fertile but seem unable to correctly germinate of their own accord. It would be useful to examine the arrangement of the mutant pollen; are the generative and vegetative nuclei correctly positioned and does the generative cell divide to produce the two sperm cells. This could be achieved by staining mutant pollen with nuclear label such as DAPI and observing the cell under a fluorescent microscope.

Examination of *Narcissus* pollen showed that a homologue of AtCAP1 was closely associated with F-Actin in the dehydrated grain (see Section 4.8). Other Actin binding proteins (including members of the ADF family) have been shown to associate with F-Actin in a similar manner (Smertenko *et al* 2001). AtCAP1 does however differ from the ADF's in that it does not appear to associate with any Actin based structures in the germinating pollen tube. It has been hypothesised that the F-Actin networks of pollen grains serve to store the monomer in preparation for germination, which requires a highly active and dynamic cytoskeleton in order to promote tip growth (Zonia 2000). It is therefore proposed that members of the CAP family bind stabilised F-Actin in the pollen grain but interactions in the pollen tube are of a transient nature due to the dynamic nature of the system. This results in a failure to detect the interaction via the immuno-localisation method used in this study. Interestingly the *Lilium* ADF that was found to bind F-Actin in pollen tubes was also found to be significantly less active (i.e. it was less able to depolymerise F-Actin) than other higher plant ADF's (Smertenko *et al* 2001). Therefore its interaction with F-Actin would be likely to occur over a longer timescale than would be expected for a more active protein so resulting in easier detection. (as shown in Gungabissoon *et al* 1998). The interaction between CAP family members and stabilised F-Actin may be under some form global control as the *Narcissus* CAP homologue did not decorate Cytochalasin D-induced Actin spicules (see Figure 4.15) Therefore it is proposed that there is a fundamental change in CAP's ability to associate with F-Actin once the pollen grain begins to germinate.

6.3.1 The Association of AtCAP1 with Actin.

The interaction between G-actin and the Carboxyl-terminal of AtCAP1 has already been clearly demonstrated (Barrero *et al* 2002) but the proteins capacity to sequester monomers was unknown and so a co-sedimentation assay was performed. There is of course a danger that using animal actin in these experiments may lead to artificial results, however plant actin was not available in suitable quantities and prior published work has successfully examined similar interactions using animal actin (Gungabissoon *et al* 1998). It was surprising to find that AtCAP1, in conjunction with F-Actin entered the pellet; this is considered strongly indicative of an ability to bind Actin filaments. The inability of AtCAP1 to inhibit polymerisation is also surprising, as other members of the CAP family have been shown to successfully sequester the monomer when present at inappropriate concentrations (Gottwald *et al* 1996). It would be expected that AtCAP1, when present at an equal concentration to Actin, would completely inhibit the formation of F-Actin. It is proposed that AtCAP1 has a lower affinity for G-Actin than other members of the CAP family, although the Yeast Two Hybrid experiments (Figure 5.5) and the work of Barrero *et al* (2002) demonstrates that such an interaction does occur. Measurement of the affinity of AtCAP1 for G-Actin would be an extremely useful experiment to perform and could be achieved via the use of Surface Plasmon Resonance technology.

The interaction between AtCAP1 and F-Actin is of interest as it suggests a potential mechanism by which the CAP-complex (as proposed by Balcer *et al* 2003) may interact with Actin filaments in higher plants. CAP family members in other kingdoms are believed to interact with F-Actin via association with APB1p (Freeman *et al* 1996), but the *Arabidopsis* genome lacks a close homologue of this F-Actin binding protein (Hussey *et al* 2002). A direct interaction between AtCAP1 and F-Actin would compensate for this absence.

The observed filament bundling activity of AtCAP1 (see Section 5.4) was also surprising as such a property has not previously been attributed to a member of the CAP family. It is unexpected because the ability to induce bundles is not an activity that would be associated with a protein believed to

promote monomer turnover (as is the case with other CAP family members). It is possible that this result demonstrates a capability unique to the higher plant CAPs but is also conceivable that the apparent bundling activity of AtCAP1 is an artefact induced by the non-physiological nature of the experiment. It is not expected that the intracellular concentration of AtCAP1 approaches that of Actin (although it has not been measured; this would be a useful experiment) and it is thought extremely unlikely that it would ever be exposed to filaments in the total absence of other actin-binding proteins. It is thought possible that the bundles shown in Figure 5.3 are result of AtCAP1 self-associating and consequently bringing the filaments to which it had also bound into contact with each other.

The relevance of the result would be demonstrated if the *in vivo* association of AtCAP1 and Actin bundles were to be shown. This could be achieved by staining sections from a cell with the anti-AtCAP1 antibody coupled with a colloidal Gold-conjugated secondary antibody and viewing the samples under a Transmission Electron Microscope.

6.3.2 The Multimerisation of AtCAP1

The Yeast Two Hybrid data presented in Figures 5.4 and 5.5 suggests that AtCAP1 is able to self-associate as both nutritional reporter genes were activated when full length AtCAP1 was present in both binding orientations. Additionally Figure 5.4 shows that the N-terminal of AtCAP1 was able to associate both with itself and with the full length-protein. These results indicate that the self-association of AtCAP1 is mediated through its N-Terminus. It would be of interest to determine whether or not the C-terminus of AtCAP1 was also able to associate with either itself or with the full length protein, as such a role has been shown for yeast CAPs (Hubberstey *et al* 1996). An attempt was made to confirm the Yeast Two Hybrid multimerisation experiments using a chemical cross-linking technique similar to that used by Ksiazek *et al* (2003) to confirm the multimerisation of *Dictyostelium* CAP. The results proved to be highly variable and so are not included in this thesis although examples are shown in Appendix IV. An alternate approach would involve the use of column gel filtration followed by western blotting in an attempt to isolate larger protein complexes containing AtCAP1.

6.3.3 The Association of AtCAP1 with Other Actin Binding Proteins .

The AtCAP1 clone was tested against selected components of the Actin Cytoskeleton in a series of Yeast Two Hybrid interaction tests; these results were presented in Section 5.6. The interaction between AtCAP1 and Actin has already been discussed in Section 5.3.1 and AtCAP1's self-association was discussed in the previous section. Potential interactions with other Actin binding proteins shall be discussed here.

It can be seen that the tests gave a very strong indication of an interaction between AtCAP1 and AtAIP1; some sign of binding could be seen in both orientations and from both reporter assays. The vegetative Profilin AtPFN2 showed evidence of interaction when it was in the Binding domain whereas the AtADF2 test only showed growth when AtADF2 was in the Activating Domain. Even then the interaction was only detectable in the lower stringency assay. The floral Profilin AtPFN3 was not found to interact with AtCAP1 in any of the assays, despite being able to bind Actin (see Figure 5.6); this demonstrates that the protein was at least partially functional when expressed in yeast.

These results suggest that AtCAP1 may be a constituent of a complex similar to that proposed by Balcer *et al* (2003) and Mattila *et al* (2004). The role of AtCAP1 in promoting filament dynamics will be discussed more fully in Section 5.4 but its inability to bind with the pollen Profilin (AtPFN3) is curious given the importance of the Actin cytoskeleton to pollen development and germination. It is possible that AtCAP1 interacts with some of the other *Arabidopsis* floral Profilins (such as PFN 4) or that Profilin and AtCAP1 do not interact within pollen tubes. This would be an extremely difficult interaction to test *in vivo* (outside of the Yeast Two Hybrid system) given that both proteins are believed to have a cytoplasmic distribution. It is possible that an advanced co-localisation technology such as Fluorescence Resonance Energy Transfer (FRET) could be used to detect such an interaction.

In vitro biochemical studies would be of use in order to reinforce the results obtained from the Yeast Two Hybrid interaction tests in this study. Such experiments should include the co-sedimentation of other Actin binding proteins with AtCAP1 and F-Actin and the study of complex formation via gel filtration. In addition the ability of individual members of the complex to interact with each should be assessed by native gel analysis. This technique was used by

Smertenko *et al* (2001) to demonstrate the interaction between G-Actin and a member of the ADF family.

6.4 The Role of AtCAP1 in Actin Dynamics

The synergistic and antagonistic interactions between Actin-binding proteins directly influence the ability of the Actin system to form polymers and higher order structures such as meshes and gels. The *in vivo* effects of AtCAP1 upon the cellular Actin network were inferred from the results of obtained using a CAP-deficient mutant and were discussed in Sections 6.2.2 and 6.2.3. The study of the complete system is hampered somewhat by both its complexity and by our incomplete understanding of it. It is therefore thought best to model the role of AtCAP1 by considering its role in terms of it's biochemical activity.

A series of studies (described in Chapter) by researchers in the yeast and animal fields have culminated in the model proposed by Balcer *et al* (2004). This was illustrated in Figure 1.11 but in essence it was proposed that large multimeric complex of CAP subunits interacted with F-Actin via an association with ABP1p. The CAP complex served to increase the rate of Actin monomer turnover by removing monomers from ADF/Cofilin family members (and thereby relieving the inhibition of monomer exchange) and passing them onto Profilin, which would actively promote nucleotide exchange and the addition of the monomer to the barbed end of the filament,

This study has demonstrated that AtCAP is able to directly associate with F-Actin and therefore compensate for the absence of ABP1p from the *Arabidopsis* genome. There is also some evidence that AtCAP1 is able to associate with ADF and some members of the Profilin family, although both interactions need to be confirmed by *in vitro* biochemical studies. It is believed likely that there are significant differences between the Higher plant CAP complex and yeast system. Primarily it has been demonstrated plant Profilins are unable to promote monomer nucleotide exchange (Perelroizen *et al* 1996b) and so it is unclear how the passing of monomers from AtCAP1 to Profilin would promote monomer addition. A quantative assessment of the affinities of the complex members for G-Actin would be of use is assessing the

likelihood that the monomer is shuttled between complex members is the expected order. Also the ability of complex members to promote polymerisation would be of interest; it is possible that Profilins are more able to mediate the transfer of monomers to the barbed end of the filament than AtCAP1. The insertion of the complex (in whole and by component) into a polymerisation assay (in conjunction with pre-formed filaments) could potentially demonstrate the importance of the complex in promoting dynamics and highlight its most important components

The interaction of AIP1 with AtCAP in Yeast Two Hybrid Experiments suggests that the interaction between the two proteins is conserved between yeast (Drees *et al* 2001) and higher plants. It is unlikely that this association would promote monomer addition as AIP is believed to act synergistically with ADF in order to promote de-polymerisation (Okada *et al* 2002). This is believed to be achieved by capping the barbed end and preventing the re-addition of monomers liberated from the filament by ADF. On possibility is that AtCAP1 is able to bind AIP and ADF when de-polymerisation of a filament is required and Profilin when polymerisation is required. The de-polymerisation of filaments by ADF/AIP/CAP would of course provide additional monomer for polymerisation elsewhere in the cell.

The association with CAP may serve to increase the affinity of other members of the complex for F-Actin by providing an additional (indirect) F-Actin binding site. This could be assessed by comparing the affinity of AIP, ADF and Profilin for F-Actin in the presence or absence of AtCAP1. Such measurements could be made by quantifying the disassociation constants ($K_{D)}$ of the proteins via co-sedimentation assays or by directly measuring the strength of the interaction with Surface Plasmon Resonance Technology.

6.5 Future Directions.

The regulation of AtCAP1 and factors affecting its interaction with Actin of great importance in understanding the *in vivo* role played by the CAP complex. Which factors affect its ability to bind other members of its complex or have a bearing upon its interaction with F-Actin? The investigation of its pH

response and the effect of small phospholipid derived signalling molecules should be the first factors investigated. Other candidate members of the CAP complex, such as ADF, are regulated by the prevailing cellular pH (Gungabissoon *et al* 1998) and the interaction between other CAP family members and G-Actin is entirely disrupted by PIP_2 (Gottwald *et al* 1996). The analysis presented in Section 3.3 suggests that AtCAP1 is likely to be a target of phosphorylation and identification of the responsible kinases and the effect of the phosphorylation upon function would be of interest. A complete yeast two hybrid screen using AtCAP1 as bait would be one potential method of identifying interacting kinases.

Such a screen would also be of use in identifying other AtCAP1-binding proteins, such as 14-3-3 proteins or perhaps more importantly candidate adenylate cyclases. This study contains relatively little regarding the potential involvement of AtCAP1 with cAMP signalling but the potential use of AtCAP1 to identify components of an almost entirely unknown pathway has not escaped the authors attention. Additionally any involvement between AtCAP1 and adenylate cyclases would render the SALK 112802 insertion line a very useful tool in the investigation of links between cAMP signalling and Actin dynamics. Direct quantification of cAMP levels within SALK 112802 Homozygotes would be an excellent first experiment.

The ability of AtCAP1 to compensate for some of ABP1p's functions (i.e. the ability to bind F-Actin) raises the possibility of other functional homologies between the two proteins. The knockout of ABP1p is yeast does not lead to an identifiable phenotype but an ABP1p/Fimbrin double mutant showed significant growth defects (Adams et al 1993). It would be of great interest to see whether or not AtCAP1 was able to complement this yeast mutation; a positive result may implicate the higher plant CAPs in contributing to vesicle trafficking.

6.6 Closing Thoughts.

This thesis represents a broad study into the nature and function of AtCAP1, a higher plant member of the CAP family. The work presented is wide reaching in both the range of techniques used and the number of questions it seeks to answer. Such an approach was believed to be appropriate for the first comprehensive attempt to study the role played by this protein in higher plants.

The key finding of this study is the importance of the CAP family to Actin organisation within higher plant cells and the relevance of this to cell expansion.

Appendix I: Primers.

Fragment Cloning

CAP.Gateway.Forward

GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAAGAGGATTTGATTA
GGCGCCC

CAP.Gateway.Fragment.Reverse

GGGGACCACTTTGTACAAGAAACGTGGGTTTAAGCCAATGCAGATAAACTA
TC

Full Length Cloning

CAP.Gateway.Forward

GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAAGAGGATTTGATTA
GGCGCCC

CAP.Gateway.Reverse

GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTAGGCACCTGAATGCG
A

Clone Sequencing

CAP.Sequence.One

CGCGTCTCCGATCCATCGATTC

CAP.Sequence.Two

GGAAGAAAGTTGGCAAATGGCTG

CAP.Sequence.Three

GGATGATATGAAGACAAAGAAC

CAP.Sequence.Four

GCTCCCACAGTTTCTGTGG

GENOTYPING PRIMERS

SALK112802.Forward

CTCCTGACTTCGCCATC

SALK112802.Reverse

GCAACCTGAACAAGTAACTATA

SALK.TDNA

GCGTGGACCGCTTTGCTGCAACT

635.Forward

CAGGATGAAATTCAGATTTATCG

635.Reverse

CTAATGATGATGGCGGAAGTCA

635.TDNA

TAGCATCTGAATTTCATAACCAATCTCGATACAC

GABI.Forward

CTCGTACCAGTAAACCGGCTTTTC

GABI.Reverse

TTGATTTGGTCTTGTTATAGCTG

GABI.TDNA

CCAATTTGGACGTGAATGTAGACAC

Appendix II: DNA and Protein Sequences

AtCAP1 (At4g34490)

ATGGAAGAGGATTTGATTAAGCGCCTTGAAGCTGCGGTTACGAGGCTTGAGGGGATCTCA
AGCAACGGAGGAGGAGTAGTTTCTCTTTCCCGCGGAGGAGATTTCTCCTCCGCCGCCGGA
ATCGATATCGCGTCGTCCGATCCATCGATTCTGGCTTATGAAGATCTGATTTCTCAATGT
GTTGGTAGGGCTCTGACCGCCGCTGAGAAAATCGGTGGACCTGTTCTAGATGTGACTAAG
ATAGTCGCCGAAGCTTTTGCTTCCCAAAAGGAGCTGCTTGTTCGCATCAAGCAAACTCAG
AAGCCTGACCTCGCTGGATTGGCTGGATTTCTCAAACCGTTGAATGATGTTACAATGAAA
GCTAATGCAATGACTGAAGGAAAAAGGTCGGATTTTTTCAATCACTTGAAGGCTGCATGC
GATAGTTTATCTGCATTGGCTTGGATTGCTTTCACTGGGAAAGATTGTGGTATGAGCATG
CCAATAGCTCATGTGGAAGAAAGTTGGCAAATGGCTGAGTTTTACAACAATAAGGTTCTG
GTGGAGTATCGTAACAAAGACGCAGATCATGTGGAGTGGGCTAAAGCCTTAAAAGAACTT
TATTTACCTGGTTTAAGGGAATATGTCAAAAGTCATTACCCCTTGGGACCTGTATGGAAT
GCATCAGGGAAACCTGCTAGTGCTCCTGCAAAGGGTCCACCTGGTGCTCCTGCTCCTCCA
CCAGCACCCCTCTTCAGTGCTGAATCTTCAAAGCCATCATCATCGTCAAACCAGAAACAA
GGGATGTCTGCTGTTTTCCAGCAACTCAGCTCGGGTGCTGTGACCTCAGGTCTTAGAAAA
GTGACGGATGATATGAAGACAAAGAACCGTGCTGATAGATCTGGAGCTGTTAGTGCGGTT
GAGAAGGAAACTCGTACCAGTAAACCGGCCTTTTCGAAAACTGGACCACCGAAAATGGAA
CTTCAAATGGGTCGCAAGTGGGCTGTTGAGAACCAAATTGGGAAGAAGGACTTGGTTATC
AGCGAGTGTGATTCCAAACAGTCTGTGTACATATATGGTTGCAAAGATTCTGTCTTGCAA
ATACAAGGAAAAGTGAATAACATCACCATTGACAAATGCACGAAAGTGGGTGTTGTTTTC
ACGGATGTTGTTGCTGCATTTGAGATTGTGAATTGCAACAACGTAGAAGTGCAATGTCAG
GGTTCAGCTCCCACAGTTTCTGTGGACAACACAACTGGCTGTCAGTTATATCTAAACAAA
GACTCATTAGAGACAGCTATAACAACAGCCAAATCAAGTGAGATCAATGTAATGGTGCCC
GGTGCTACCCCTGATGGAGATTGGGTGGAACATGCGCTGCCTCAACAGTACAATCATGTG
TTTACTGAAGGGAAGTTTGAGACAACACCGGTCTCGCATTCAGGTGCCTAA

AtCAP1

MEEDLIKRLEAAVTRLEGISSNGGGVVSLSRGGDFSSAAGIDIASSDPSILAYEDLISQCVGRALTAAEK
IGGPVLDVTKIVAEAFASQKELLVRIKQTQKPDLAGLAGFLKPLNDVTMKANAMTEGKRSDFFNHLKAAC
DSLSALAWIAFTGKDCGMSMPIAHVEESWQMAEFYNNKVLVEYRNKDADHVEWAKALKELYLPGLREYVK
SHYPLGPVWNASGKPASAPAKGPPGAPAPPPAPLFSAESSKPSSSSNQKQGMSAVFQQLSSGAVTSGLRK
VTDDMKTKNRADRSGAVSAVEKETRTSKPAFSKTGPPKMELQMGRKWAVENQIGKKDLVISECDSKQSVY
IYGCKDSVLQIQGKVNNITIDKCTKVGVVFTDVVAAFEIVNCNNVEVQCQGSAPTVSVDNTTGCQLYLNK
DSLETAITTAKSSEINVMVPGATPDGDWVEHALPQQYNHVFTEGKFETTPVSHSGA

Expressed Fragment (The His Tag and Linker are Underlined)

MGSSHHHHHHSSGLVPRGSQSTSLYKKAGLMEEDLIKRLEAAVTRLEGISSNGGGVVSL
SRGGDFSSAAGIDIASSDPSILAYEDLISQCVGRALTAAEKIGGPVLDVTKIVAEAFAW
QKELLVRIKQTQKPDLAGLAGFLKPLNDVTMKANAMTEGKRSDFFNHLKAACDSLSALA

Appendix III: The Optimisation of AtCAP1 Production.

The method used for the *in-vitro* production of AtCAP1 in an E.coli host was detailed in Section 5.2. The protocol was developed and optimised to yield the greatest amount of relatively pure AtCAP1 in a stable form. Initially it was found that a reasonable amount of AtCAP1 (approximately 0.4mg $L^{-1}$of culture) could be produced using the BL21-DE3 strain of expression cell in LB media with 6 hours of induction prior to harvesting. A typical elution profile is shown bellow in Figure A3.1:

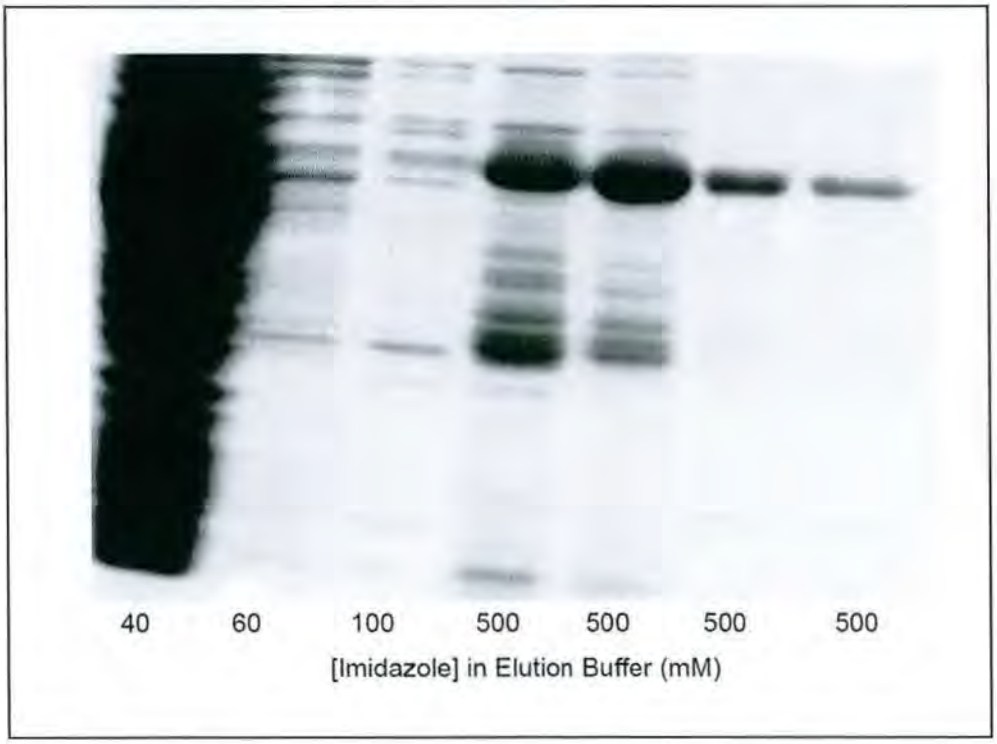


Fig A3.1: The Elution of bacterially expressed AtCAP1 from a Hi-trap column. (Each lane corresponds to 1 ml of flow-through).

It can be seen that a relatively pure band is present following the prior application of 2 ml of 500mM Imidazole elution buffer. Unfortunately, a considerable amount of AtCAP1 was lost in the first 2 ml of elution. This loss was significantly reduced by re-loading the entire 500mM Imidazole elution onto a Hi-trap column and inserting a 200mM wash prior to the final elution. The additional wash removed many of the contaminants meaning that all fractions of the 500mM wash could be used.

Alternate strains of *E.coli* were used in an attempt to increase the final yield of the AtCAP1 construct. The BL21-pLysS strain expresses a Bacteriophage T7 Lysoszyme that inhibits the activity of the T7 RNA polymerase used within pET-based expression systems (pGAT4 is a derivative of pET28a). This reduces basal (i.e. prior to induction) expression of the construct and so prevents potentially toxic expression-proteins from inhibiting bacterial growth (Studier 1991). In this case, the use of BL21-pLysS did not increase the yield of AtCAP1, which suggests that the construct was not toxic to the expression system. The BL21-pLysS-Rosetta strain was also tested in an attempt to overcome any disparity in codon-use between the *Arabidopsis* source of the gene and the *E.coli* expression system. The seven additional tRNA's conferred by the Rosetta plasmid did have previously been shown to aid the expression of some Eukaryotic genes in *E.coli* hosts (Dabrowski and Kiaer-Ahring 2003). Again, this did not increase the yield of AtCAP1.

The length and temperature of induction were also varied in a final attempt to increase the amount of AtCAP1 produced. Reducing the temperature to 30^0C had no effect upon the amount of protein generated although increasing the induction time did (see Figure A3.2 below).

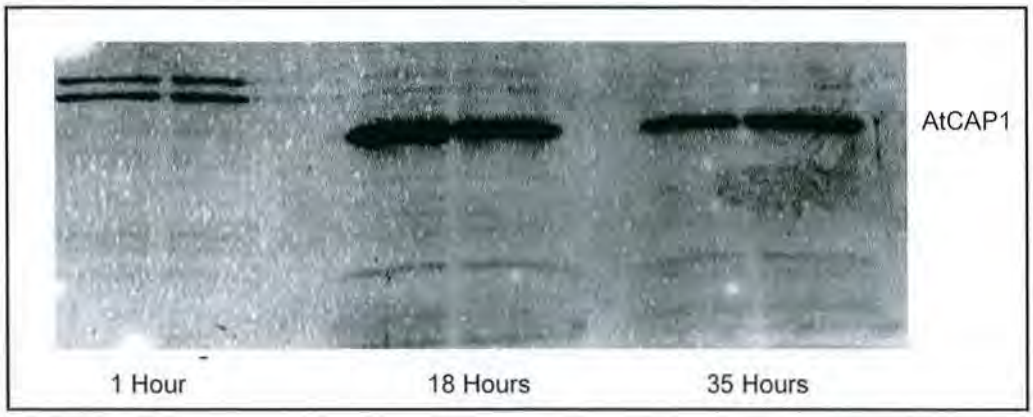


Fig A.3: A Time-Course for AtCAP1 Expression.

It can be seen that bands corresponding to AtCAP1 are not present in the sample taken after one hour of induction (the previous experiments had used two hours of induction time). A considerable amount of the protein was present in the sample from the 18-hour induction and this had reduced 35 hours post-induction (presumably due to degradation). The 18-hour induction was used throughout the rest of the experiments and would regularly provide

1 mg L^{-1} of culture. Variation of the measured optical density (from 0.1 to 1.5) prior to induction was found to have no effect upon the final yield.

Section 2.2.4.3 detailed how an additional ultra-centrifugation stage was used prior to co-sedimentation assays. Initially this was found to remove most of the AtCAP1 produced, leaving far too little for experimentation. A series of empirical experiments led to the development of a method of handling CAP that dramatically increased the useable amount. Firstly it was found that exchanging the buffer via dialysis (8 hours with three changes of buffer at 4°C) greatly increased the survivability of the CAP (previously a de-salting column had be used). Secondly, the addition of 10% glycerol to whichever buffer the CAP was to be dissolved into (usually PBS or G-Buffer) also decreased the amount lost to aggregation/precipitation during preparation.

Appendix IV: Cross-linking of AtCAP1.

Previous studies have demonstrated that non-animal CAP's are able to bind each other via their N-terminus (Ksiazek et al 2003) and that such interactions may be demonstrated via Chemical Cross-linking. An attempt was made to replicate the results achieved Ksiazek et al (2003) using full length AtCAP1 as opposed to N-terminal fragment as it was thought that such an experiment would be both more interesting and more straightforward (the AtCAP1 N-terminal fragment was extremely unstable).

Initially the method of Ksiazek was used, i.e. AtCAP1 was dialysed into a pH 7.0 HEPES buffer with 20mM NaCl and diluted to 0.5 mg ml^{-1} . N-hydroxylsulposuccinimide (NHS) was added to a concentration of 5 mM for 10 minutes at room temperature prior to the addition 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCL (ECL) to a concentration of 10 mM. This cross-linking reaction proceeded at room temperature for 3 minutes before it was terminated by the addition of protein sample buffer (as described in Section 2.2.3.3). Samples of the cross-linking reaction were then run on a SDS-PAGE gel; the formation of CAP-CAP dimmers (or higher order complexes) would have been evidenced by the appearance of a higher molecular-weight band. It can be seen in Figure A4.1 that no such band is present.

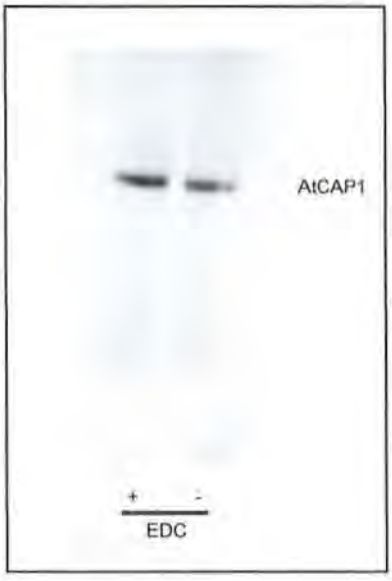


Fig A4.3: An EDC cross-linking experiment. The lane on the left shows the result of the cross-linking whereas the lane on the right contains an EDC-free control.

This experiment was repeated several times and on each occasion a negative result was obtained. Similar results were achieved when the concentration of AtCAP1 was varied and when the cross-linking time was increased to 30 minutes.

A further series of experiments were performed where the concentration of the cross-linking agent, i.e. EDC was varied in a final attempt to cause linkage. This is shown in Figure A4.2 below and again it can be seen to be have been unsuccessful. It is impossible to draw any conclusions regarding the multimerisation of AtCAP1 from these results as potential positive controls also gave negative results (data not shown). As such, this lack of cross-linking may be a false negative or may be indicative of *full-length* AtCAP1 not interacting with itself under these conditions.

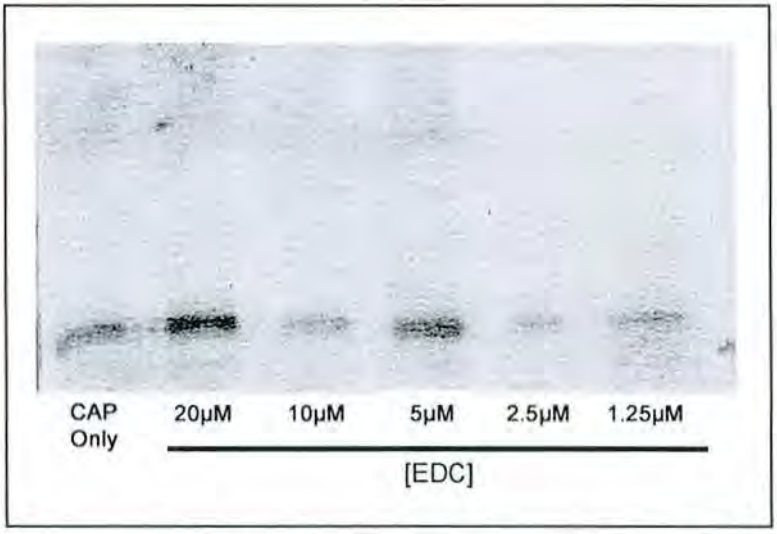


Fig.A4.2: Variations in EDC concentration do not lead to AtCAP1 cross-linking.

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