Understanding the Role of the Arp2/3 Complex and its Upstream Regulator in Actin Cytoskeleton Mediated Organization of the Endoplasmic Reticulum in Plant Cells

by

Madhulika Sareen

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Molecular and Cellular Biology

Guelph, Ontario, Canada

© Madhulika Sareen, May, 2013
ABSTRACT

UNDERSTANDING THE ROLE OF ARP 2/3 COMPLEX AND ITS UPSTREAM REGULATOR IN ACTIN CYTOSKELETON MEDIATED ORGANIZATION OF THE ENDOPLASMIC RETICULUM IN PLANT CELLS

Madhulika Sareen                                                    Advisor: Dr.Jaideep Mathur
University of Guelph, 2013

The Actin Related Protein (ARP) 2/3 complex is a major regulator of the actin cytoskeleton that is implicated in cell morphogenesis in plants. However, a similar role is attributed to the endoplasmic reticulum (ER). My research explored the relationship between the two systems by using transgenic plants simultaneously expressing fluorescent proteins highlighting F-actin and ER organization in living cells. A comparison of F-actin organization in cells of wild type Arabidopsis thaliana and mutants with aberrant actin cytoskeleton suggests bundling in the distorted2 mutant but a relatively fine F-actin arrangement in klunker. These differences correlate with ER organization into cisternae, fenestrated sheets and tubules. A model relating ER-organization to the degree of actin bundling in a cell emerges and is supported by drug-induced interference in actin polymerization, altered ionic conditions and temperature. The study adds to the mechanistic understanding of cell morphogenesis in plants.
Acknowledgements

The project would be incomplete without me acknowledging everyone who helped me tremendously throughout the tenure of my project.

I convey my deepest gratitude to my advisor, Dr. Jaideep Mathur and his valuable time, guidance and support at every stage of my research. His instructions, kindness and patience with me have enabled me to learn a lot. I would like to thank my committee members Dr. John Greenwood and Dr. John Dawson for their time and valuable comments on the thesis, helping me improve and learn.

I would like to thank Mrs. Neeta Mathur for not only taking care of my plants but also me, for all her motivating and priceless suggestions, guiding me through the difficult times.

I am indebted to my lab mates Martin, Firas, Kati, Alena, Kiah, Michael and Ashley for their valuable inputs, suggestions, moral and intellectual support.

I am grateful to my parents, for their continued support, encouragement and love. Last but not the least my friends for helping me overcome setbacks, maintaining my focus throughout this time and the Lord, for his many blessings.
Table of Contents

Chapter 1: Introduction........................................................................................................... - 1 -

1.1 The Cytoskeleton...................................................................................................................... - 1 -

1.1.1 Actin cytoskeleton modulated by actin interacting proteins........................................ - 1 -

1.1.2 The Arp2/3 complex and its upstream regulatory complex...................................... - 6 -

1.1.3 Mutations in the genes of Arp2/3 complex and its upstream regulator SCAR/WAVE complex ........................................................................................................ - 10 -

1.1.4 Subcellular defects in the mutants of the Arp2/3 complex and its upstream regulator .................................................................................................................................. - 14 -

1.2 The endomembrane system.................................................................................................- 17 -

1.2.1 The endoplasmic reticulum and its modulators ......................................................... - 19 -

1.2.2 Link between ER and the actin cytoskeleton............................................................... - 20 -

1.3 Hypothesis........................................................................................................................... - 25 -

1.4 Experimental Approach...................................................................................................... - 25 -

1.5 Cell biological fluorescent protein-based tools................................................................. - 29 -

1.5.1 The use of fluorescent protein probes for understanding sub-cellular dynamics ........................................................................................................................................ - 29 -

Chapter 2: Materials and Methods ........................................................................................... - 34 -

2.2 Transgenic and mutants plants ............................................................................................. - 34 -
2.3 Sample preparation for epifluorescence, confocal and scanning electron microscope .......................................................... - 35 -

2.3.1 Epifluorescence microscopy ................................................... - 35 -

2.3.2 Confocal Microscopy .............................................................. - 36 -

2.3.3 Scanning Electron Microscopy ............................................... - 36 -

2.4 Molecular Biology ...................................................................... - 37 -

2.4.1 Preparation of competent Agrobacterium tumefaciens .............. - 37 -

2.4.2 Agrobacterium transformation-by electroporation method .......... - 37 -

2.4.3 Arabidopsis transformation-floral dip method ......................... - 38 -

2.5 Plant stress treatments ............................................................... - 39 -

2.5.1 Cold stress ........................................................................... - 39 -

2.5.2 Latrunculin-B treatment .......................................................... - 39 -

2.5.3 Phosphate stress treatment ...................................................... - 40 -

2.6 Post-acquisition Image processing ............................................ - 40 -

Chapter 3: Results ........................................................................ - 42 -

3.1 Creation of tools ........................................................................ - 42 -

3.2 General morphological observations on the mutants ................... - 44 -

3.3 General observations of the mutants: F-actin organization .......... - 49 -

3.4 Morphological characterization of endoplasmic reticulum in distorted2 and klunker ................................................................. - 53 -
3.5 Understanding the morphological dependence of endoplasmic reticulum on the F-actin organization. ................................................................. - 59 -

3.6 Observing the effect of several factors like actin interacting drugs, cold stress and phosphate starvation on ER morphology and behaviour........................... - 66 -

Chapter 4: Discussion........................................................................................................ - 80 -

4.1 Actin organization dependent change in ER morphology of distorted2 and klunker.................................................................................................................. - 80 -

4.2 Factors disrupting the actin organization also influence shape and behaviour of ER in wt Arabidopsis hypocotyl cells.................................................. - 87 -

4.3 Conclusion .............................................................................................................. - 91 -

4.4 Future prospects ................................................................................................... - 92 -

5. References ............................................................................................................. - 94 -

Appendix A- Recipes................................................................................................. - 104 -

Appendix B- Abbreviations ...................................................................................... - 108 -
List of Tables

Table 1.1: Actin binding proteins and their functions.

Table 1.2: Some cellular defects recognized as a result of compromised Arp2/3 complex activity in different organisms.

Table 1.3: Gene Names and basic properties of the known Arabidopsis Arp2/3 Complex and WAVE complex subunits.

Table 1.4: Mutants with defects in ER morphology and network.

Table 2.1: Origin of Arabidopsis mutants.

Table 2.2: Origin of fluorescent protein probes

Table 3.1: Lines created for each of the actin organization mutants and control (Ler) transformed with GFP::FABD2 mTalin, RFP::HDEL and CX::mEosFP.
List of Figures

Figure 1.1: Diagrammatic representation of the polymerization process producing F-actin from G-actin monomers.

Figure 1.2: Multiple branching of actin filaments seen in the form of a dendritic array at the leading edge of lamellipodia.

Figure 1.3: Model of actin filament branching mediated by the Arp2/3 complex in Acanthameoba.

Figure 1.4: Diagrammatical representation of cell shape defects present in the mutants with actin organization defects.

Figure 1.5: Figure shows actin configuration in epidermal hypocotyl and cotyledon pavement cells of mutants and wt.

Figure 1.6: Schematic showing the step by step advancement of the project.

Figure 1.7: Arabidopsis wild type seedling.

Figure 3.1: Scanning electron micrographs of epidermal hypocotyl and petiole cells from 10 day old dark grown seedlings.

Figure 3.2: Scanning electron micrographs of trichomes from 10 day old light grown wt and mutants.

Figure 3.3: Histogram showing the hypocotyl length differences between the wt and mutant.

Figure 3.4: Histogram depicting the differences in the average hypocotyl epidermal cell surface area between wt and mutant.

Figure 3.5: Histogram depicting the differences in the average hypocotyl epidermal cell length between the wt and mutant.

Figure 3.6: F-actin organization in the hypocotyl epidermal cells of 7-10 day old seedlings of wt and mutant.

Figure 3.7: Cortical endoplasmic reticulum visualized in the hypocotyl epidermal cells of wt and mutants.

Figure 3.8: Diagrammatic representation depicting the differences in the endoplasmic reticulum between the wt and mutant.

Figure 3.9: Histogram depicting the variation in the percent surface area covered by ER membrane in wt and mutants.

Figure 3.10: Histogram showing differences in the ER polygons between wt and mutants.
Figure 3.11: Histogram revealing deviations in the ER body number and area between wt and mutant.

Figure 3.12: Simultaneous visualization of F-actin and ER in the hypocotyl epidermal cells of wt and mutant.

Figure 3.13: Diagrammatical representation of actin and ER arrangement in wt and mutants.

Figure 3.14: Simultaneous visualization of F-actin and ER in the hypocotyl epidermal cells of the distorted2.

Figure 3.15: Simultaneous visualization of F-actin and ER in the hypocotyl epidermal cells of distorted2.

Figure 3.16: Endoplasmic reticulum in the hypocotyl epidermal cells of the 12 day old seedlings in control and Lat-B treated seedlings.

Figure 3.17: Graph displaying morphological differences in the ER polygons of Latrunculin-B treated wt seedlings.

Figure 3.18: Graph displaying differences in the average individual cisternae surface area of Latrunculin-B treated wt and control.

Figure 3.19: Endoplasmic reticulum in the hypocotyl epidermal cells of 12 day old wt seedlings at different growth temperature.

Figure 3.20: Graph displaying morphological differences in the ER polygons under normal and cold stress conditions wt seedling.

Figure 3.21: Graph displaying percent surface area of the ER membrane under normal and cold stress conditions wt seedlings.

Figure 3.22: Arabidopsis wt seedlings grown on MS media with different phosphate concentrations.

Figure 3.23: Endoplasmic reticulum in the hypocotyl cells of 12 day old seedlings grown on MS media with different phosphate concentrations.

Figure 3.24: Graph showing morphological differences in the ER polygons wt seedlings are grown on MS media with different phosphate concentrations.

Figure 3.25: Graph showing average percent surface area occupied by the ER membrane under phosphate stress conditions.
Chapter 1: Introduction

1.1 The Cytoskeleton

The cytoskeleton comprises an arrangement of filamentous polymers and acts as a major receiver and transducer of diverse environmental signals for the eukaryotic cell (Wasteneys and Galway, 2003; Wasteneys and Yang, 2004; Mathur, 2007). Microtubules and microfilaments are the main cytoskeleton elements in a plant cell. They constantly undergo remodeling and fulfill roles in supporting cell division, cell enlargement and differentiation. Microtubules are long, hollow, unbranched polymer tubes assembled from α and β-tubulin heterodimers that are arranged in rows or proto-filaments. In contrast, microfilaments are relatively solid, thinner structures composed of the protein actin that are often organized into a branching network (Karp, 2009).

1.1.1 Actin cytoskeleton modulated by actin interacting proteins

Actin exists in the form of a globular 42 kDa monomeric unit known as G (globular)-actin which polymerizes to form long filaments known as F-(filamentous) actin. The polymerization proceeds with a rapid hydrolysis of an ATP molecule bound to G-actin, producing F-actin (Straub and Feuer, 1950). The resulting actin filament is helical and polarized with one end, called the barbed end (newest end) containing the ATP bound monomers and the other, older end (pointed end) with ADP bound monomers. The whole process proceeds spontaneously and shapes the actin cytoskeleton organization within a cell (Figure 1.1).
Actin filaments are known to organize into a variety of patterns including bundles and cables of varying thickness, thin 2-dimensional networks and complex 3-dimensional gels (Karp, 2009). This complex organization and behavior of F-actin inside a cell is determined by a number of actin binding proteins (ABPs) that affect the assembly and disassembly of actin, their physical interaction with one another and with other cellular organelles.

![Diagram of actin polymerization](image)

**Figure 1.1:** Diagrammatic representation of the polymerization process producing F-actin from G-actin monomers. Each individual actin monomer is bound by a molecule of ATP which gets rapidly hydrolysed following polymerization. The initial phase in the creation of filamentous actin involves the association of globular actin monomer subunits resulting in a dimer and a trimer formation, which are rather slow steps. Eventually, the assembly of G-actin monomers results in the formation of F-actin. The rapid hydrolysis of ATP bound to the G-actin monomers creates polarity in the actin filament such as the positive end (barbed end) contains ATP bound actin monomers and the negative end (pointed end) contains ADP bound actin monomers (based on figure 1 from May, 2001).

Functions performed by these ABPs include tracking the pool of actin monomer subunits available for polymerization, recognizing the site of actin nucleation and
polymerization, controlling the rate of filament assembly and disassembly and organizing actin filaments into higher order structures (McCurdy et al., 2000). A general grouping of various actin binding proteins and their functions is presented in Table 1.1. The foremost step in *de novo* actin filament creation is actin nucleation resulting in an actin trimer. The energetically unfavorable process involving the creation of a nucleus and actin filament polymerization becomes favorable in the presence of certain actin binding proteins. The Actin Related Protein (Arp) 2/3 complex and formins are two major ABPs found in plants which are known to regulate the process of actin polymerization or de-polymerization by binding to globular actin and enhancing or preventing its ability to add to filaments (Machesky et al., 1994; Winder et al., 2005).

Table 1.1: Actin binding proteins and their functions

<table>
<thead>
<tr>
<th>ABPs</th>
<th>Arabidopsis Homologs</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arp2/3 complex (7-subunits)</td>
<td>1</td>
<td>Actin nucleation and polymerization (Cvrckova, 2012; Mathur, 2005)</td>
</tr>
<tr>
<td>Formins</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Profilin</td>
<td>5</td>
<td>Actin polymerizing and de-polymerizing (Wang et al., 2009; Ruzicka et al., 2007; Deeks et al., 2007)</td>
</tr>
<tr>
<td>Actin de-polymerizing factor (ADF)/Cofilin</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Cyclase associated protein (CAP)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fimbrin</td>
<td>5</td>
<td>Actin bundling and cross-linking (Wu et al., 2010; Zang et al., 2010)</td>
</tr>
<tr>
<td>Villin</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Myosins</td>
<td>17</td>
<td>Actin used as a physical support or track (Reddy et al., 2001)</td>
</tr>
</tbody>
</table>
These two ABPs differ in their mechanism of action in creating new actin filaments. In contrast to the Arp2/3 complex, formins function by associating themselves to the fast growing barbed end of the actin filament enabling rapid insertion of the actin subunits. Formins are characterized by the presence of a conserved formin homology domain2 (FH2) (Zigmond, 2004), along with a proline rich formin homology domain1 (FH1) required for actin nucleation. There are 21 formins identified in Arabidopsis and these have further been classified into two groups on the basis of presence or absence of an N-terminal trans-membrane domain (Higaki et al., 2007; Blanchoin et al., 2010).

Once nucleated by the Arp2/3 complex or formins, in principle the actin filaments can continue growing with the addition of actin monomers at the barbed ends. At this stage several other ABPs come into play that bind with either the G-actin or F-actin, altering their assembling properties and dynamics. Profilin, ADF (actin de-polymerizing factor)/Cofilin and CAP (cyclase associated protein) are the three major protein families that bind to the G-actin and enhance its ability to add to actin filaments.

Profilin, one of the actin monomer binding proteins, facilitates actin polymerization onto free/uncapped filament ends under suitable conditions. It also suppresses spontaneous nucleation of new actin filaments and prevents the assembly of these actin monomers at the pointed ends (Staiger et al., 2006). The activity of profilin is regulated by calcium (Ca\(^{2+}\)) concentration. It also binds to polyphosphoinositides and proline rich proteins, both of which are implicated in signaling events that result in the reorganization of actin (Gibbon and Staiger, 2000).
ADF (Actin de-polymerizing factor)/cofilin consists of a family of actin severing proteins (McCurdy et al., 2000) that are also involved in increasing the dissociation rate of actin subunits from the pointed ends and phosphate release after polymerization (Staiger et al., 2006). ADF/cofilin activity is regulated by changes in the pH, phosphorylation status and polyphosphoinositides (Blanchoin et al., 2010). To date, 11 ADF/ cofilin-like actin-binding proteins have been reported in Arabidopsis (Ruzicka et al., 2007).

Another known set of ABPs arranges actin filaments into higher order structures. These proteins are involved in either actin bundling, cross linking or stabilizing the filaments. Fimbrins and villins belong to this group of actin binding proteins (ABPs). Fimbrins are a highly conserved family of actin filament bundling proteins (McCurdy et al., 1998). They have two actin binding domains (ABD1 and ABD2), each composed of a tandem repeat of calponin homology domains together with two helix loop helix motifs. There are five fimbrin like genes identified in Arabidopsis (Staiger and Hussey, 2004). Villins belong to the gelsolin/ severin family of ABPs and are characterized by the presence of six gelsolin/severin domains and a C-terminal headpiece domain for binding to microfilaments (Higaki et al., 2007). To date, 5 villin-like genes have been identified and characterized in Arabidopsis (Klahre et al., 2000; Huang et al., 2005).

Other actin binding proteins use actin as a physical support or track. Myosins belong to this class of actin dependent molecular motors, producing movement through the hydrolysis of ATP. They consist of three domains; the highly conserved N-terminal domain that binds to actin filaments through the hydrolysis of ATP; the IQ motif that
binds to calmodulin or calmodulin related proteins and a tail domain consisting of alpha helices responsible for protein dimerization. There are 17 myosin-like genes identified in Arabidopsis, classified into two families; class VIII and class XI (Reddy et al., 2001). Classes VIII myosins contain 4 genes and the gene products localize to the plasma membrane and plasmodesmeta (Reichelt et al., 1999), while class XI myosins consist of 13 genes involved in organelle movement (Peremyslov et al., 2012), such as the movement of endoplasmic reticulum (Ueda et al., 2010), peroxisomes (Hashimoto et al., 2002), mitochondria (Verbelen et al., 2002), chloroplast (Sattarzadeh et al., 2009) and golgi bodies (Peremyslov et al., 2008).

1.1.2 The Arp2/3 complex and its upstream regulatory complex

The Arp2/3 complex is considered a major player in the process of actin nucleation and polymerization and was first isolated from Acanthameoba using profilin, an actin binding protein, through ion exchange chromatography (Machesky et al., 1994). The Arp2/3 complex can nucleate filaments from the side of existing filaments at a precise angle of 70° (Mullins et al., 1998). In this way it creates a dendritic network, as is seen at the leading edge of motile cells (Figure 1.2) (Svitkina and Borisy, 1999). The complex is composed of seven subunits with two large actin like subunits Arp2 and Arp3. The other five subunits of the complex are named as Arpc1/p41, Arpc2/p31, Arpc3/p21, Arpc4/p20, and Arpc5/p16. Figure 1.3 shows the molecular model of the actin bound Arp2/3 complex at a branch junction.
The Arp2/3 complex has been implicated in several processes among different organisms primarily through defects observed due to the mutations in different subunits of the complex or a general malfunctioning of the complex (Table 1.2).

Figure 1.2 : Multiple branching of actin filaments seen in the form of a dendritic array at the leading edge of lamellipodia (based on figure 1 from Parsons et al., 2010).

The activity of the Arp2/3 complex in vivo is enhanced by functional interactions with members of the Wiskott Aldrich Syndrome (WASP) family of proteins. There are
two principal classes of proteins present in the WASP family; SCAR/WAVE and WASP. The SCAR/WAVE protein family class carries a dual name as it was discovered simultaneously in two independent studies (Bear et al., 1998, Miki et al., 1998). Both SCAR/WAVE and WASP share a conserved C-terminal VCA (Verproline homology Connecting Acidic domain) catalytic domain (Millard et al., 2004), which helps in bringing G-actin and the Arp2/3 complex together and leads to the nucleation of a branched filament. However, these protein complexes differ in the signaling input they receive and the way they are regulated. WASP is an auto-inhibited protein, and a physical interaction with the small GTPase cdc42 and phosphatidylinositol 4,5-bisphosphate allows it to activate the Arp2/3 complex.

Figure 1.3: Model of actin filament branching mediated by the Arp2/3 complex in Acanthameoba. Shown in the figure is a filamentous actin behaving as backbone onto which the Arp2/3 complex (seen in red) binds at an angle of 70° and initiates a new actin filament. The barbed end of the filament is towards the top of the figure and the pointed end is towards the bottom of the figure (based on figure3a from Volkmann et al., 2001).
SCAR/ WAVE, in contrast, is not auto-inhibited and forms a part of a large regulatory complex that contains four other proteins, namely PIR121, NAP1, Abi and HSPC300 (Eden et al., 2002). In addition the organization of the N-terminal domain which provides connections with the regulatory proteins differs among the WASP protein family members (Takenava and Suetsugu, 2007). The WASP protein consists of a WASP homology1 domain and a CRIB domain, helping to bind the small GTPase cdc42 (Symons et al., 1996; Rohatgi et al., 1999) while the SCAR/WAVE complex contains a SCAR homology domain and lacks the GTPase binding domain (Bear et al., 1998).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Salient Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td>Shape defects: Mutations in ARP2, ARP3, ARPC2, ARPC5 lead to random shape alterations in epidermal cells due to misdirected expansion. Display defects in actin organization.</td>
</tr>
<tr>
<td>Budding Yeast</td>
<td>Shape defects: Mutants in ARPC1/ non-viable. Mutants in other subunits conditional; usually display defects in cortical actin cytoskeleton. Mutants in ARPC5 and ARP3 subunits exhibit aberrant mitochondrial behavior.</td>
</tr>
<tr>
<td>C.elegans</td>
<td>Motility defect: RNAi mediated depletion of different subunit leads to defects in ventral closure.</td>
</tr>
<tr>
<td>Drosophila</td>
<td>Motile cell behavior defective: Loss-of-function mutants frequently embryo/juvenile stage lethal. Affect blastoderm organization, ring canal expansion, axon development and eye morphogenesis.</td>
</tr>
<tr>
<td>Fission yeast</td>
<td>Shape defects: Mutants in ARPC1/ non-viable. Mutants in other subunits conditional; display vacuolation and actin cytoskeleton defects. Defects in contractile ring formation during cytokinesis.</td>
</tr>
<tr>
<td>Mammals</td>
<td>Motile cell behavior affected: RNAi of the ARPC3 subunit is lethal in mouse.</td>
</tr>
</tbody>
</table>
Homologs of all the five subunits of the SCAR/WAVE complex have been identified in Arabidopsis (Szymanski et al., 2005). According to a model proposed by Millard et al., (2004) the association of SCAR1 with HSP3000 through its VCA domain is usually kept repressed by PIR121, Abi2 and SRA1. With the activation and binding of Rac-GTPase to the pentameric complex, SCAR- HSP3000 is released and the rest of the complex activates the Arp2/3 complex resulting in actin filament nucleation and polymerization (Millard et al., 2004).

Table 1.3 modified from Szymanski et al., (2005) lists known Arabidopsis Arp2/3 complex and WAVE complex subunits. DIS3 encodes a plant specific SCAR/WAVE complex and is suggested to assemble into a WAVE complex via physical interaction with Arabidopsis Abi1 like bridging protein (Zhang et al., 2005; Basu et al., 2005).

1.1.3 Mutations in the genes of Arp2/3 complex and its upstream regulator SCAR/WAVE complex

A large collection of Arabidopsis mutants created through EMS mutagenesis, affecting different aspects of trichome morphogenesis was reported by Huelskamp et al., (1994). Out of these, eight genes (ALIEN, CROOKED, DISTORTED1, DISTORTED2, KLUNKER, GNARLED, SPIRRIG and WURM ) were grouped into a “distorted” class on the basis of characteristic distorted trichome phenotype shown by each of the mutants (Oppenheimer et al.,1993; Huelskamp et al.,1994).

The distorted trichome phenotype of these mutants was justified by several suggested reasons such as defects in the biosynthetic machinery of the cell wall components,
improper trafficking of the newly synthesized material to the final destination and changes in the intracellular or vacuolar properties (Oppenheimer et al., 1993; Hulskamp et al., 1994). In addition, two independent studies (Mathur et al., 1999; Syzmanski et al., 1999) successfully mimicked the distorted trichome phenotype by treating seedlings of Arabidopsis thaliana with actin disrupting drugs like cytochalasin-D, latrunculin-B, phalloidin, and jasplakinolide. These actin disrupting drug-based studies hinted towards a possible link between the ‘distorted’ class of genes and the maintenance and regulation of the actin cytoskeleton.

Table 1.3: Gene Names and basic properties of the known Arabidopsis Arp2/3 Complex and WAVE complex subunits (based on Szymanski et al., 2005)

<table>
<thead>
<tr>
<th>Name of the mutant</th>
<th>Chromosome no</th>
<th>AtDB Ac.No</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wurm</td>
<td>4</td>
<td>AT3G2700</td>
<td>ARP2</td>
</tr>
<tr>
<td>Distorted1</td>
<td>1</td>
<td>AT1G13180</td>
<td>ARP3</td>
</tr>
<tr>
<td>Distorted2/hsr3</td>
<td>1</td>
<td>AT1G30825</td>
<td>ARPC2</td>
</tr>
<tr>
<td>Crooked</td>
<td>3</td>
<td>AT4G04710</td>
<td>ARPC5</td>
</tr>
<tr>
<td>Gnarled</td>
<td>2</td>
<td>AT2G35110</td>
<td>NAP1</td>
</tr>
<tr>
<td>Klunker</td>
<td>5</td>
<td>AT5818410</td>
<td>SRA1</td>
</tr>
<tr>
<td>Arpc4</td>
<td>4</td>
<td>AT4G14147</td>
<td>ARPC4</td>
</tr>
<tr>
<td>Atbrk1</td>
<td>2</td>
<td>AT2G22640</td>
<td>BRICK1</td>
</tr>
<tr>
<td>Dis3</td>
<td>2</td>
<td>AT2G38440</td>
<td>SCAR2</td>
</tr>
</tbody>
</table>

Later studies identified and molecularly characterized four of the ‘distorted’ class genes distorted1 (Mathur et al., 2003), distorted2 (Saedler et al., 2004, El-Assal et al.,
2004), *wurm* (Mathur et al., 2003), and *crooked* (Mathur et al., 2003), encoding plant homologs ARP3, ARPC2, ARP2, and ARPC5 (smallest subunit of Arp2/3 complex), respectively. The mutant alleles contained single point mutations, causing an amino acid change in the protein sequence further affecting the generation, activity or stability of the protein. For instance, the single point mutation at position 1040 at the splice donor site of the second exon lead to premature splicing, a 35bp deletion in exon 2 and a frameshift, resulting in a stop codon 129 bp from the start codon (Mathur et al., 2003a). An allele of the *ARPC2* locus has recently been identified and designated as *hsr3* (*HIGH SUGAR RESPONSE3*) (Jiang et al., 2012). Another ‘distorted’ class gene *SPIRRIG* was shown to encode a WD40/BEACH domain containing protein involved in membrane trafficking and membrane composition events (Saedler et al., 2009).

These mutants of the Arp2/3 complex (*wurm, distorted1, distorted2, crooked*) showed high phenotypic similarities concerning trichomes, pavement cells of the cotyledons, first leaves and root hairs (Mathur, 2005). The trichomes are short and stumpy with unextended branches in these mutants. A single report mentions finding a distorted trichome phenotype in a T-DNA insertion mutant line for the *ARPC5/p16* gene of the Arp2/3 complex (Lord et al, 2003). When challenged to grow under dark conditions, the mutants display hypocotyls with elongating epidermal cells breaking contact with their neighboring cells and curling out in unsynchronized growth (Mathur et al., 2003a,b). The root hairs of mutants (*crooked, wurm, distorted1*) show a stunted and wavy growth pattern (Mathur et al., 2003a; b).
Among the distorted trichome class of mutants, four (wurm, distorted1, distorted2, crooked) were shown to encode subunits of the Arp2/3 complex, indicating that proteins regulating the actin cytoskeleton could cause distorted trichomes. Further studies identified and molecularly characterized two other distorted class genes as parts of the WAVE/SCAR protein complex. GNARLED, encodes for a NAP125/NAP1 (El-Assal et al., 2004; Brembu et al., 2004) (Nck associated protein 1) homolog while KLUNKER/PIROGI (klunker/pir), encodes a PIR121 (p53-121F-induced) / SRA1 (Specifically RAC1 associated) homolog (Basu et al., 2004). The distorted trichome phenotype is seen in the T-DNA knockouts of both NAPP and PIRP (PIR121 of plants; Brembu et al., 2004), indicating their role in the organization of actin cytoskeleton. Similarly, mutations in other subunits of the Arabidopsis SCAR/WAVE complex, BRICK1 (Djakovic et al., 2002) and DIS3 (Basu et al., 2005) genes result in trichome distortions, pavement cell morphology defects and defects in cell-cell adhesion. Displayed in figure 1.4 are the defects in different cell types of the mutants (distorted1, distorted2, klunker, gnarled, crooked and wurm) in Arabidopsis.

The moss Physcomitrella patens also reflects the critical role played by the SCAR- Arp2/3 in cell shape development and growth. In the Physcomitrella moss, mutants in the Arp2/3 and SCAR/WAVE complex subunits show severe cell morphological defects with ‘irregularly shaped cells and abnormal division pattern’ (Harries et al., 2005) causing a drastic decrease in filament growth (Perround and Quatrano, 2008) and producing shortened cubic chloronemal cells (Finka et al., 2008). Moreover the localization of BRICK1 (the subunit of the SCAR/WAVE complex) and ARPC4 to the
filament apex in the moss reflect the critical role of SCAR-Arp2/3 in the promotion of tip growth (Perround and Quatrano, 2006, 2008).

1.1.4 Subcellular defects in the mutants of the Arp2/3 complex and its upstream regulator

Defects in actin organization are clearly observed in ‘distorted’ class mutants (Mathur et al., 1999; Szymanski et al., 1999). Except for trichomes in gnarled and klunker mutants, the trichomes in other mutants showed thick aggregates of bundled F-actin in the form of patches with dispersed pockets containing a diffuse network. Even the cotyledon cells and the cells of the hypocotyls had abnormal accumulations of bundled with thick F-actin filaments.
Figure 1.4: Diagrammatical representation of epidermal cell shape defects present in the mutants with actin organization defects (distorted2, distorted1, klunker, gnarled, arpc4, crooked, wurm). The mutant trichomes appear in a distorted form, the pavement cells show a deviation from original jigsaw shape with gaps appearing in between the adjacent cells, the hypocotyl cells loose contact with their adjoining neighbors and curl out, root hairs show a wavy growth pattern sometimes producing more than one growth tip (Figure modified from Mathur, 2005)

This pattern of F-actin distribution in mutants is suggested to create unequal and random regions of expansion and localized bulging. It was suggested that as a consequence of intense F-actin bundling in the mutants, the delivery of vesicles carrying cell wall building material was abnormal and caused unbalanced growth of the cells
(Mathur et al., 2003a). An impairment of membrane fusion capability was speculated in *wurm* and *distorted1* mutants where a large number of un-fused vacuoles were observed vs. one large central vacuole in the wild type plants (Mathur et al., 2003b). Furthermore, the *crooked* mutants exhibit significantly reduced rate of cytoplasmic streaming in areas of intense actin bundling with less expansion. Trichomes in *crooked* also showed an increase in the number and size of Golgi bodies, as compared to wild type plants where the Golgi bodies were regularly distributed. The extensive actin bundles also cause a subsequent reduction in the organelle (Golgi and peroxisome) movement in the non-expanded regions of *crooked* mutant (Mathur et al., 2003a). Another study, reported guard cell insensitivity to external stimuli as a result of bundled F-actin in the *distorted2* allele *hsr3* mutants (Jiang et al., 2012).

While the mutants of the Arp2/3 complex were characterized by excessive F-actin bundles, *gnarled* and *klunker*, which are a part of the SCAR/WAVE complex, distinctly exhibit ‘fine actin’ configuration (Mathur et al, 1999). Similar studies stated a resemblance between the *gnarled* and wild type actin bundles (El-Assal et al., 2004) with the average ratio of core to total actin in mutants similar to the wild type (Li et al., 2004). Differences were apparent in the way actin was organized in these mutants. While in the wild type, F-actin cables are long and aligned along the direction of branch growth, *Arabidopsis* SCAR/WAVE mutants *gnarled, klunker* (Li et al., 2004) and *dis3* (Basu et al., 2005) have more disorganized bundles of F-actin. However, these findings were inconsistent with another report where the *gnarled* and *klunker* mutants showed an abnormal accumulation of F-actin, forming thick bundles which are transversely oriented (Brembu et al., 2004). Other mutants of the *Arabidopsis* SCAR/WAVE complex, *brick1*,
reported an absence of diffuse actin at developing cell lobes (Djakovic et al., 2003). Figure 1.5 represents the actin organization in different cell types seen in mutants wurm, distorted2, crooked, and klunker.

In moss Physcomitrella patens, dense converging arrays of F-actin bundles were reported at the growing tips of protonema cells. However, these converging arrays of F-actin bundles vanished when treated with actin depolymerizing drugs cytochalasin-B and latrunculin-B further affecting the cellular tip growth (Finka et al., 2007). Similarly, disappearance of specific F-actin cortical structures responsible for localized cell growth were reported due to loss of function mutations of Arp3a in P.patens (Finka et al., 2008).

1.2 The endomembrane system

The endomembrane system is made up of a set of interconnected membranes lying within a eukaryotic cell. The membranes divide the cell into structural and functional units known as organelles. These organelles can communicate directly through contacts or indirectly through targeted vesicles (Hadlington and Denecke, 2000) and non-vesicular transport (Lev et al., 2010). Different subcellular components that form a part of the endo-membrane system are the nuclear envelope, endoplasmic reticulum, Golgi bodies, trans-golgi network, multivesicular bodies, endosomes, peroxisomes, prevacuolar compartments (PVCs), vacuole, vesicles and the plasma membranes (Fujimoto and Ueda., 2012). The endoplasmic reticulum (ER) forms a major part of the endomembrane system.
Figure 1.5: Figure shows actin configuration in epidermal hypocotyl and cotyledon pavement cells of mutants with actin organization defects in comparison to wt.

(A) wt (B) wurm (C) dis2 cotyledon pavement cells. Pavement cells of wt Arabidopsis seedlings, with well defined pavement cell lobes possess fine F-actin organization. While the mutant wurm and dis2 shows bundled actin formation in the cotyledon pavement cells with no apparent lobes.

(D) wt (E) dis2 (F) klk (G) crk epidermal hypocotyl cells. Dark grown elongated wt hypocotyl cells show a fine F-actin meshwork. Thick actin bundles in a reticulate form observed in dark grown crooked, dis2 epidermal hypocotyl cells. Arrow points towards the gaps appearing between the neighboring hypocotyl epidermal cells in dis2. Epidermal hypocotyl cell in klk, appear to have a fine actin organization.
1.2.1 The endoplasmic reticulum and its modulators

The ER is disseminated throughout the cell in the form of distinct sub-domains distinguished by their unique structure and function. These sub-domains include the outer membrane of the nuclear envelope, cortical ER, the cytoplasmic ER as well as specific sub-domains associated with various organelles (Staehelin, 1997; Sparkes et al., 2011). The cortical ER is arranged as a network of dynamic polygons of extending and retracting tubules lying under the plasma membrane. Flattened sheets of ER called ER cisternae are present between some of these polygons. In root and hypocotyl cells of Arabidopsis thaliana, the ER cisternae is more frequently observed in expanding as compared to mature, fully expanded cells (Ridge et al., 1999).

The dynamic remodeling of the cortical ER network is influenced by factors regulating the extension and retraction of cortical ER tubules, the branching of these tubules as well as their relative stability over time. A class of ER membrane proteins in yeast and mammals, known as reticulons (RTN), are required for maintaining the high curvature of ER tubules (Voeltz et al., 2006). Membrane curvature is the result of the wedge shaped trans-membrane topology which is conserved amongst the designated structural motif of the reticulon homology domain. Reticulon homologs from non-chordate taxa are divided into six reticulon-like (RTNL) protein subfamilies where the subfamily specific to plants is RTNLB. In A. thaliana, 21 RTNLB proteins have been identified (Nziengui et al., 2007). In particular, RTNLB1-4 localizes to the high curvature membrane of ER tubules and is excluded from the lamellar sheets (Sparkes et al., 2010). When over-expressed, RTNLB13, similar in localization as RTNLB1-4, causes increased
ER tubule constriction and decreased diffusion of soluble proteins throughout the ER lumen (Tolley et al., 2008).

While reticulons are responsible for the structure of ER tubules, another class of ER membrane-bound, dynamin like GTP binding proteins known as atlastins help to interconnect ER tubules to create the complex ER network (Hu et al., 2009). In *A. thaliana*, RHD3 (ROOT HAIR DEFECTIVE 3) has been classified as an atlastin GTPase (Hu et al., 2009). RHD3 resides on the ER tubules and ER punctae, and like other atlastins, is involved in the generation of a tubular ER network (Chen et al., 2011).

A recent study has proposed synchronized action of both atlastins and reticulons to shape the ER in plant cells (Lee et al., 2012). The disordered tubular ER observed through the co-expression of RTNLB13 and RHD3 in tobacco leaf epidermal cells, was created through additive effect of RHD3 and RTNL13. This interaction between the RHD3 and RTNLB is also supported by co-immunoprecipitation studies (Lee et al., 2012). Along with *rhd3*, several other mutants showing defects in the ER morphology and networking have been isolated and characterized in *Arabidopsis* (Table 1.4).

### 1.2.2 Link between ER and the actin cytoskeleton

In animal cells, the dynamic behavior of the ER and its intracellular positioning is regulated by both microtubules (Terasaki et al., 1986) and microfilaments (Liebe et al., 1995; Sparks et al., 2009; Staehelin, 1997). However, in plants, the dynamics of the endomembrane system appears to depend largely on actin filaments rather than microtubules. Actin filaments are regulated and maintained by a number of actin binding
proteins. The Arp2/3 complex is one of the major regulators of the actin cytoskeleton, promoting actin nucleation and polymerization in a plant cell. Several actin interacting drugs like Latrunculin-B (Lat-B) compete with G-actin monomers, possibly limiting the activity of the Arp2/3 complex and preventing F-actin assembly.

Actin disrupting drugs have been used to probe the consequences of disrupting ER tubule extension-retraction behavior without disturbing the overall structure of the ER (Sparkes et al., 2011). With the application of actin disrupting drugs, ER dynamics cease and the “ER showed accumulation in the form of patches, fusions of tubules into cisternae and an overall shape change demonstrating a loss of pulling force” (Knelbel et al., 1990). “Large lamellar islands” have also been reported in the cortical ER network in Vallisneria gigantean cells with another actin disrupting drug (Liebe et al., 1995). Similar drug treatments in Nicotiana tabacum (Runions et al., 2006) and the epidermal cells of cotyledon petioles of A. thaliana have resulted in the cessation of ER remodeling along with a decrease in protein flow within the ER lumen (Ueda et al., 2010). In addition, an alteration in organization, shape and distribution of ER was observed in onion bulb epidermal cells in consequence to cold treatment. It was suggested that low temperature affects the interaction of actin and myosin which in turn affects the ER organization (Quader et al., 1989).
Table 1.4: Mutants with defects in ER morphology and network.

<table>
<thead>
<tr>
<th>Name of the Mutant</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ermo1</strong></td>
<td>ERM01 encodes GNOM LIKE1 (GNL1), a cis-golgi-localized ARF-GEF (guanine nucleotide exchange factor for ADP-ribosylation factor). ARF-GEFs activate ARF, a small GTPase, which recruits various effector proteins. GNL1 is involved in activating ARF proteins which initiate the formation of COP1 vesicles for retrograde transport from golgi to ER. The <em>ermo1</em> mutants develop a number of spherical bodies that are about 1 um in diameter and dispersed throughout the cell (Nakano et al., 2009).</td>
</tr>
<tr>
<td><strong>ermo2</strong></td>
<td>ERM02 encodes SEC24a, which is a member of coat protein complex responsible for transport from ER to golgi. The <em>ermo2</em> mutants develop a number of spherical bodies of the size of about 1 um diameter which are present in the form of large aggregates and also found dispersed along the periphery of the cell (Nakano et al., 2009).</td>
</tr>
<tr>
<td><strong>nai1</strong></td>
<td>NAI1 is a bHLH protein that could function as a transcription factor. In plants bHLH proteins are known to be involved in several physiological processes. There is an absence of ER bodies in <em>nai1</em> mutant (Matsushima et al., 2004).</td>
</tr>
<tr>
<td><strong>nai2</strong></td>
<td>NAI2 encodes a unique protein, the homologue of which is found only in the Brassicaceae plants. Reduction in Nai2 levels reduces the number of ER bodies and alters their shape. <em>nai2</em> mutant shows complete deficiency of ER body(Yamada et al., 2008).</td>
</tr>
<tr>
<td><strong>rhd3</strong></td>
<td>RHD3 is a large GTP-binding protein originally identified in a screen for root hair defective mutants (Wang et al., 1997). The cortical ER in the <em>rhd3</em> mutants show loss of three way junctions and formation of large cable like structures (Chen et al., 2011).</td>
</tr>
<tr>
<td>kam1/murus3</td>
<td>kam1/murus3 Katamari1/murus3</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>KAM1 is a type II membrane protein. The kam1 mutant shown defects in the organization of endo-membranes and actin filaments (Tamura et al., 2005).</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>kam2 Katamari2</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAM2 is a homolog of animal receptor mediated endocytosis8 (RME-8) proteins that are involved in endocytosis. The kam2 mutants show aggregates of endomembrane structures, deformed endosomal compartments and defects in vacuolar transport in seed cells (Tamura et al., 2007).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pah1/2 Phosphatidic acid phosphohydrolase1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pah1/pah2 are Mg$^{2+}$-dependent phosphatidic acid phosphohydrolases in Arabidopsis thaliana acting redundantly to repress phospholipid biosynthesis at the endoplasmic reticulum. pah1/pah2 double mutant exhibits a reduction in galactolipids along with a dramatic increase in the level of phospholipids leading to overexpansion of ER membrane (Eastmond et al., 2010).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ermo3/mvp1/gold36 Endoplasmic reticulum morphology3/Modified vacuole phenotype3/Golgi defects 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERMO3/MVP1/GOLD36 is a member of GDSL lipase/esterase family also known to interact with PYK10 complex, a complex thought to be important for ER body related defense system. The mutant ermo3/mvp1/gold36 is characterized by the formation of huge aggregates, abnormal punctate structures of ER along with aberrant intercellular distribution of most organelles(Nakano et al., 2012).</td>
</tr>
</tbody>
</table>

In combination with lat B, which inhibits the addition of G-actin to the growing actin filaments, visual maps reflecting the degree of local persistence of the cortical ER in N. tabacum have been used to further underscore the effect of actin disruption on ER dynamics (Sparkes et al., 2009). As expected, the number and size of cisternae increased with Lat-B treatment. Using persistency maps the rate at which ER tubules, their polygon ring structure, ER punctae and cisternae rearrange themselves was shown to slow down.
Accordingly Sparkes et al., (2009) suggested a model for the involvement of actin in ER formation and reorganization where persistent ER punctae act as anchor/growth sites for the extension and retraction of ER tubules. The extension-retraction activity of ER tubules may occur along the actin microfilaments through actin polymerization. It was suggested that in the presence of actin disrupting drugs, the acto-myosin system may become nonfunctional. As a consequence, polygon branch sites may start become unstable with the inner sides of the tubule filling up to cause cisternalization. Hence, the increased number and size of persistent cisternae can be linked to the presence of non-functional actin polymerization machinery. Notably, the actin polymerization is believed to be impaired in the Arp2/3 complex mutants, too, but it is not known whether persistent cisternal islands also appear in these mutants.

Conversely, there are also mutants that exhibit defects in both ER and actin organization. The Arabidopsis rhd3 mutants show altered actin organization where filaments are often bundled together to form thick cables (Hu et al., 2003). The rhd3 mutants also show defects in ER resulting in loss of three way junctions and formation of large cable like structures, suggesting its role in the generation of tubular ER network (Chen et al., 2011). However, it is not known whether RHD3 physically interacts with actin filaments to control the tubular ER network in plant cells. As a matter of fact, it is unclear whether the ER phenotype observed in the rhd3 mutants is created as a consequence of bundled actin or the less branched ER affects the conformation of the actin cytoskeleton. A similar Arabidopsis mutant, katamari1 (kam1), has defects in actin filaments and forms endo-membrane aggregates within the cells. These endo-membrane
aggregates are suggested to be caused by the formation of aggregates of actin cytoskeleton in \textit{kam1} (Tamura et al., 2005).

Work using actin disrupting drugs suggests a strong correlation between the actin and ER organization behavior. Alternatively, there are also mutants like \textit{kam1} and \textit{rhd3}, where simultaneous defects in the actin and ER configuration are observed. Literature has confirmed the role played by the actin cytoskeleton in shaping the ER; however, how the actin polymerization factors like Arp2/3 may also be involved in mediating ER organization is still not clear.

1.3 Hypothesis

Previous research has suggested an interplay between F-actin organization and endoplasmic reticulum morphology and dynamics. However, there is no information available on whether the Arp2/3 complex, a primary F-actin nucleation and polymerization factor, is directly involved in mediating F-actin dependent ER organization.

I hypothesize that \textit{The Arp2/3 complex, through its effect on actin organization, is involved in organizing the ER and therefore mutations in different subunits of the complex and its upstream regulatory complex will exhibit an abnormal ER organization”}

1.4 Experimental Approach

A pre-requisite for carrying out experiments to provide evidence supporting the aforementioned hypothesis, involved creation of new cell biological tools and
characterization of different cell types for carrying out the investigations. As shown in schematic figure 1.6, the cell biological tools consisted of mutants with actin organization defects, stably transformed with genes encoding the fluorescent probes for the endoplasmic reticulum and F-actin and double transgenic mutants that combine genes encoding both probes. Based upon the ability to look inside the cells at the F-actin, different organ and cell types (hypocotyl, cotyledon, trichomes) in wild type and mutant backgrounds were characterized. Further on, epidermal cells from the middle region of the hypocotyl as indicated in Figure 1.7, were chosen to carry out detailed observations on the endoplasmic reticulum and F-actin arrangement in klunker and distorted2.
Figure 1.6: Schematic showing the step by step advancement of the project starting from the stage of creating transgenic tools to the stage of observation and analysis.
Figure 1.7: *Arabidopsis* wild type seedling. Labeled are the various organs of the *Arabidopsis* seedlings. Figure points towards the hypocotyl which is the stem of the germinating seedling, found below the cotyledons and above the radical. Cotyledons are the embryonic first leaves of the seedling. Petiole is the stalk of cotyledon or first true leaves attaching the blade to the stem. Trichomes are the unicellular, stellate, epidermal hairs present on the first true leaves.
1.5 Cell biological fluorescent protein-based tools

Studies in the past have strongly suggested a role for the actin cytoskeleton in organizing the endoplasmic reticulum in plants (Sparks et al., 2009; Liebe et al., 1995; Knelbel et al., 1990). Most of the work was conducted with the help of actin disrupting drugs that either stabilize F-actin filaments or prevent actin polymerization. Alternatively it has been possible to microinject fluorescently labeled phallotoxin in living plant cells and observe their actin organization. However, this method is labor intensive, prone to wounding, induces artifacts and the chemical stabilizes F-actin (Kost et al., 2000). The effects of drugs and inhibitors vary in their intensity, in the time of action and between different tissue types. In some cases they might even be non-specific. Thus it is not known how naturally-occurring but non-functional actin polymerization machinery in a cell would actually affect the organization and dynamics of the endoplasmic reticulum. A direct and relatively non-invasive approach that circumvents the use of drugs is to study mutants with impaired actin functioning and use live imaging tools for directly visualizing their subcellular organization and behavior. This approach has been used successfully in several studies on plant development (Mathur et al., 2003 a,b)

1.5.1 The use of fluorescent protein probes for understanding subcellular dynamics

Fluorescent actin filament labeling gained great importance after the development of an actin labeling fluorescent probe in 1998 with the cDNA sequence of green fluorescent protein (GFP), fused to the F-actin binding domain of the mouse talin gene (Kost et al., 1998). The probe is well established, allowing non-invasive visualization of actin
filaments in a range of different types of plant cells and tissue (Yu et al., 2001; Baluska et al., 2000; Mathur et al., 1999). In addition, an inducible mTalin gene under the control of an ethanol inducible promoter has also been created (Ketelaar et al., 2004). However, studies by Sheahan et al., (2004), Ketelaar et al., (2004) and Wang et al., (2008) have suggested that GFP-mTalin fusion proteins may cause artificial aggregation of actin networks, indicating their inability to accurately depict actin organization and turnover in plant cells. Overexpression of GFP-mTalin has also been suggested to affect the flow of auxin (Nick et al., 2009). Yet most of the reported artifacts might be due to transient overexpression (Ketelaar et al., 2004) or increased probe stability interfering with the actin dynamics (Wang et al., 2008; Schenkel et al., 2008). GFP-mTalin was used as a fluorescent probe in the current research project to label actin filaments and visualize the actin organization in the Arp2/3 mutants. In our hands, Arabidopsis transformed with GFP-mTalin behaved and grew normally without any phenotypic defects that could have been caused by actin bundling through overexpression of GFP-mTalin. At the same time, an Arp2/3 mutant carrying GFP-mTalin maintained their morphology like those of plants without GFP-mTalin, suggesting that GFP-mTalin had little or no effect on either the actin organization in a plant cell or overall plant growth and development.

A number of other actin filament visualization probes have been created with fusions between fluorescent protein probes and different actin interacting factors; for example, GFP fused to the headpiece domain of Arabidopsis villin (Klahre et al, 2000), or actin depolymerizing factor NtADF1 from tobacco fused to GFP (Chen et al., 2002). Shehan et al., (2004) created a fusion between GFP and actin binding domain 2 (ABD2) of the Arabidopsis fimbrin gene which successfully demonstrated detailed features of the actin
cytoskeleton in protoplasts derived from stably transformed mesophyll tissue. An improved version of GFP-FABD2 was also created where GFP was fused both at the N and C terminal of ABD2 (Wang et al., 2007) resulting in enhanced GFP fluorescence, helping to collect highly resolved F-actin networks in different plant organs and stages of development.

All actin interacting proteins fused to GFP, however, showed limitations of only highlighting F-actin in a single fluorescent color. This restricted the quantification of any local changes in F-actin organization within a cell, thereby also preventing the capture of any interactions of F-actin with the organelles in the vicinity. To overcome this limitation, several photo-convertible, photo-switchable and photo-activatable probes have been developed. The photo-activatable fluorescent protein probes function by the transformation of chromophore (ρ-HBI) from a non-fluorescent neutral form to a fluorescent anionic state by irradiation with intense light of 400 nm such as PA-GFP (Patterson et al., 2002). In photo-switchable probes such as PS-CFP2, cis-trans isomerisation of the chromophore is exploited for reversible switching between the bright and dark states of the chromophore (Chudakov et al., 2004). By contrast, photo-convertible fluorescent proteins function by converting the chromophore irreversibly from one colour to another, e.g., from a green to red fluorescent state, by the photochemical modification of the peptide backbone. EosFP is a photo-convertible fluorescent protein obtained from scleractinian coral Lobophyllia hemprichii that irreversibly switches its peak emission from 516 to 581 nm upon UV irradiation (Wiedenmann et al., 2004). Although the original EosFP is in a tetrameric form, it has
been engineered to a monomeric state while maintaining its high photo-convertibility and fluorescence quantum yield (Wiedenmann et al., 2004).

A new photo-convertible actin fluorescent probe has recently been developed that added high precision in labeling F-actin along with following F-actin dynamics and interactions with other organelles (Schenkel et al., 2008). A monomeric Eos-fluorescent protein was fused to the F-actin binding domain of the mammalian Talin gene to create mEosFP::FABD mTalin (Schenkel et al., 2008). Another probe was developed using a 17 amino acid peptide called LIFEACT from the yeast Abp140p LIFEACT fused to a GFP or an RFP has emerged as the smallest probe used to label actin filaments causing minimal interference with actin dynamics (Riedl et al., 2008). Using this probe as a backbone, a LIFEACT::mEosFP fusion was created. This probe has the advantage of not only being the smallest probe but also showing a high potential for estimating localized alterations in F-actin organization (Mathur et al., 2010).

Morphological insights into the dynamic nature of ER have been possible from investigations of living cells by means of video enhancement and fluorescence microscopy techniques. The first successful attempt to label and highlight the ER with GFP was made by Boevink et al., (1996) using a virus-based expression system. The signal peptide from the storage protein patatin was fused to the N-terminus of the GFP with the ER retention signal KDEL fused to the C-terminus. KDEL is a highly conserved region derived from luminal ER proteins in animal cells which is shown to be necessary and sufficient for proteins to be retained within the ER. Later on, in 1997, Haseloff and co workers successfully produced the mGFP-ER construct which is now one of the most
used probes for plant ER. The construct has an *Arabidopsis* basic chitinase at its N-terminal acting as a signal sequence and a C-terminal ‘HDEL’ ER retention sequence fused to GFP. Like KDEL, HDEL is present at the C-termini of yeast reticuloplasmins that helps in retaining the fluorescent protein signal in the lumen of ER, enabling to visualize cortical ER streaming with characteristic tubular polygonal networks and variously shaped cisternae.

A photo-convertible probe PA-GFP-ER consisting of a transmembrane domain of the *Arabidopsis* ER integral membrane protein calnexin, has been used to effectively label the ER membrane (Runions et al., 2006). PA-GFP-ER formed the basis for creating a novel fluorescent photo-convertible probe CX-mEosFP probe, (Mathur et al., 2010) which has the advantage of being bright fluorescent green before photo-activation and can be easily photo-converted into a bright red colour.
Chapter 2: Materials and Methods

2.1 Plant material preparation

Seed sterilization was carried out by first washing the seeds for 10 minutes on a vortex mixer (Fisher Scientific) in a 20% bleach solution (Clorox Ultra Bleach, Brampton, ON). Seeds were then washed for 5 times, a minute each with autoclaved ddH₂O. The sterilized seeds were then spread with the help of autoclaved ddH₂O on plates containing MS salts (Phytatechnology Laboratories, USA) with either 0.8% Phyto Agar (Duchefa Biochemie, The Netherlands) or 0.3% Phytagel (Sigma-Aldrich). The plates were wrapped in parafilm (Bemis flexible packaging; Neenah, WI) and left at 4°C in a refrigerator for 2 days. After 2 days, the plates were transferred to a growth chamber (Percival; Boone, IO) with a light dark cycle of 16 hr/8 hr and growth temperature between 20-22°C.

2.2 Transgenic and mutants plants

Table 2.1: Origin of Arabidopsis mutants

<table>
<thead>
<tr>
<th>Name of the mutant</th>
<th>AtDB.Ac.No</th>
<th>Gene name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crooked</td>
<td>At4g01710</td>
<td>ARPC5</td>
<td>Hulskamp et al., 1994</td>
</tr>
<tr>
<td>Distorted1</td>
<td>At1g13180</td>
<td>ARP3</td>
<td>Hulskamp et al., 1994</td>
</tr>
<tr>
<td>Distorted2</td>
<td>At1g30825</td>
<td>ARPC2</td>
<td>Hulskamp et al., 1994</td>
</tr>
<tr>
<td>Wurm</td>
<td>At3g27000</td>
<td>ARP2</td>
<td>Hulskamp et al., 1994</td>
</tr>
<tr>
<td>Arpc4</td>
<td>At4g14127</td>
<td>ARPC4</td>
<td>SALK T-DNA</td>
</tr>
<tr>
<td>Gnarled 247</td>
<td>At2g35110</td>
<td>NAP135</td>
<td>Hulskamp et al., 1994</td>
</tr>
<tr>
<td>Klunker</td>
<td>At5g18410</td>
<td>PIR121</td>
<td>Hulskamp et al., 1994</td>
</tr>
</tbody>
</table>
Table 2.2: Origin of fluorescently tagged probes

<table>
<thead>
<tr>
<th>Target</th>
<th>Fluorescence</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F- Actin</td>
<td>Green</td>
<td>F-actin binding domain of mouse Talin gene (amino acid number 2345-2543) fused to GFP</td>
<td>Kost et al., 1988</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>Red</td>
<td>RFP-HDEL construct created by replacing GFP in mGFP-ER (Haseloff et al., 1997) with a monomeric RFP and placed in a binary pCAMBIA vector</td>
<td>Sinclair et al., 2009a</td>
</tr>
<tr>
<td>Endoplasmic Reticulum</td>
<td>Green to Red photo-convertible</td>
<td>Trans-membrane domain of Arabidopsis Calnexin fused to photo-convertible green to red, monomeric Eos fluorescent protein</td>
<td>Mathur et al., 2010</td>
</tr>
</tbody>
</table>

2.3 Sample preparation for epifluorescence, confocal and scanning electron microscope

2.3.1 Epifluorescence microscopy

Plants grown on MS media plates were removed from the agar plates and placed on depression slides containing water and covered with a coverslip (Fisher Scientific). The plants were viewed under an epifluorescence microscope Nikon Eclipse 80i (Nikon Canada, Mississauga, ON) equipped with a 40X Nikon Plan Apochromat lens with a numerical aperture 0.95 and a Leica DFC 300FX Camera. The chroma filter sets used were Endow GFP-LP filter set 41018 (Ex, HQ 470/40X; dichroic, Q495LP; Em,
HQ500LP), tetramethylrhodamine isothiocyanate filter set 41002c (Ex, HQ545/30X; dichroic, Q570LP; Em, HQ 620/60), and the DAPI/Hoechst/aminomethylcoumarin acetate filter set 31000V2 (Ex, 350/50X; central dichroic, LP 400; Em, 460/50).

2.3.2 Confocal Microscopy

Confocal images were captured using a Leica DM6000 microscope equipped with a Leica TCS SP5 confocal scanning head. Samples were viewed under a 40X water immersion lens (numerical aperture: 0.80) and excited using either a 488 nm argon laser or 543 nm helium neon laser. The resolution of a confocal microscope depends upon the excitation wavelength \( \lambda_{ex} \) of the light being used along with the numerical aperture (NA) of the lens, stated by the formula \( 0.37 \lambda_{ex} / NA \) (Webb, 1996). Planar x/y/z images and x/y/time scans were obtained using Leica Confocal Software and saved in a 1024 X 512 pixel format. Fluorescence emission collection was at 490-510 nm for GFP, at 570-620 nm for RFP and 626-763 nm for chlorophyll.

2.3.3 Scanning Electron Microscopy

Scanning electron micrographs were captured using Hitachi TM-1000 Tabletop Microscope. 7-10 day old seedlings were directly taken from \( \frac{1}{2} \) MS basal media plates and mounted on the sample holder of the Hitachi TM-1000 Tabletop Microscope. The sample was viewed through the Hitachi TM-1000 software on a computer with Windows XP professional, for only 2-5 minutes after being placed in the vacuum chamber since the material starts dehydrating and losing its shape. The software saved the images in 1280 x 1040 pixel format.
2.4 Molecular Biology

2.4.1 Preparation of competent *Agrobacterium tumefaciens*

*Agrobacterium* competent cells were prepared according to the protocol outlined by Hofman and Willmitzer, (1988) by inoculating YEB with the *Agrobacterium* culture strain GV3101 in the presence of 100 ug/ml rifampicin (Fisher Scientific), 25 ug/ml of gentamycin (Fisher Scientific) and 50 ug/ml kanamycin. 2 ml of the overnight grown culture was added to 50 ml YEB containing the desired antibiotics and the culture was shaken vigorously at 250 rpm (New Brunswick Scientific, Edison, NJ) and 28°C-30°C. On reaching the required OD$_{600}$ (Optical density at a wavelength of 600 nm) =0.5-1.0, the culture was chilled on ice for 10 minutes and later centrifuged (Hermle Z 320 K swinging bucket centrifuge) at 3000 g for 15 minutes at 4°C. After centrifugation, the supernatant was discarded and cells were resuspended in 1 ml of 20 mM CaCl$_2$. Lastly the cells were aliquoted (100 ul) into precooled 1.5 ml Eppendorf tubes and stored at -80°C. (Sanyo ESBE Scientific, VIP series)

2.4.2 *Agrobacterium* transformation-by electroporation method

*Agrobacterium* transformation was carried out using the protocol described in Weigel and Glazebrook, (2006). The competent *Agrobacterium* strain was obtained from -80°C refrigerator (Sanyo ESBE Scientific, VIP Series).The cells were thawed on ice for about 2-3 minutes. About 3-5 ul of miniprep DNA was added to 100 ul of bacteria and transferred to a pre-chilled cuvette (1 mm Electroporation cuvettes from Molecular Bio Products). The electroporater (Biorad Pulse Controller plus Biorad gene pulser II) was set
to use at 50 µF, 1.8 kV and 200 Ω. The cuvette was then wiped using a kimwipe to remove any moisture sticking to its surface and placed in the electroporator. The toggle switch on the electroporator was used to create an electrical pulse once, with the machine displaying a time constant between 4-4.7 msec. Thereafter, the cuvette was removed and 1 ml of prechilled YEB media was added. The bacteria were transferred back to the culture tube and incubated (Fisher Isotemp Incubator, 200 Series model 255D) at 28°C for 2 hours with gentle shaking. The bacteria were then plated on selective medium and left in an incubator at 28°C for 2 days.

2.4.3 *Arabidopsis* transformation-floral dip method

To generate stably transgenic plants expressing the fusion proteins of interest, each recombinant plasmid was introduced into the *Agrobacterium tumefaciens* strains GV3101. The plasmids were then introduced into *Arabidopsis thaliana* ecotype *Landsberg erecta* (Ler) via the floral dip method, which results in transformation of female gametes (Clough and Bent, 2006). *Agrobacterium* mediated transformation of plants was done with a slight modification to the floral dip method developed by Clough and Bent, (1998). A 500 ml of overnight *Agrobacterium* culture was grown from *Agrobacterium* colonies obtained from selective medium plates, in an orbital shaker at 28°C. Bacteria were transferred to 400 ml centrifuge bottles (Nalgene) and centrifuged at 7000 rpm for 15 minutes at room temperature. The supernatant was discarded and bacterial pellet was resuspended in a resuspension medium (5% Sucrose, 50 ul/ L Silwet L-77). A magnetic stir bar was added to the bacterial resuspension and stirred slowly on a stir plate until the entire bacterial pellet was resuspended. 2 week old *Arabidopsis* plants with no siliques
were submerged in the bacterial suspension and all the flowers and buds immersed. The plant was removed after 1 minute in the bacterial suspension and kept sideways on a tray to allow excess solution to drip off. On the following day plants were placed upright in the trays, watered and maintained in the growth chamber.

Seeds were harvested from transformed plants and selected on hygromycin (20 ug/ml)-containing MS agar plates. Green seedlings were transferred to soil at about 2 rosette leaf growth stage. Each single line was carried to T2 and T3 generation before carrying out further analysis.

2.5 Plant stress treatments

2.5.1 Cold stress

½ MS basal medium plates with 8-12 day old wt *Arabidopsis* dark grown seedlings (plates wrapped in foil) were left vertically standing in a refrigerator at 4°C for 24 hours. The control ½ MS media plate with wt *Arabidopsis* dark grown seedlings (plates wrapped in foil) were left vertically standing, in the growth chamber (Percival; Boone, IO) with growth temperature between 20-22°C. Further observations were made using a Leica DM6000B microscope equipped with a Leica TCS SP5 confocal scanning head.

2.5.2 Latrunculin-B treatment

8-12 day old wt *Arabidopsis* seedlings plated on ½ MS basal media plates were treated with 500 nmol solution of Latrunculin-B (stock prepared in DMSO). Plants were removed from plates and kept in the drug solution in water for 1 hour. The control
seedlings were left in water for 1 hour. Further observations on were made using a Leica DM6000B microscope equipped with a Leica TCS SP5 confocal scanning head.

2.5.3 Phosphate stress treatment

The wt Arabidopsis seedlings were grown on MS media with three different phosphate concentrations: 170 mg/l, 340 mg/l and phosphate starvation conditions. The MS media was constructed using the formulations provided by Murashige and Skoog (1962). Since the media had to be made with varying phosphate concentrations, each constituent of the media was added separately. The list of these constituents of MS media include: Murashige and Skoog (1962) medium basal salt micronutrient solution (M0529; Sigma Aldrich, Canada), Gamborg’s vitamin solution (1000x) (G1019; Sigma Aldrich, Canada), macronutrients NH\(_4\)NO\(_3\) (1650 mg/l), CaCl\(_2\) (440 mg/l), MgSO\(_4\) \(\cdot\) 7H\(_2\)O (370 mg/l), KNO\(_3\) (1900 mg/l) added from their respective 100x stock solutions and the gelling agent used was 0.8% agarose (Bioshop, Canada). The only macronutrient added in varying accounts in each case of phosphate starvation, normal phosphate (170 mg/l) and double phosphate (340 mg/l) was potassium phosphate (KH\(_2\)PO\(_4\)). Once plated, wt Arabidopsis seedlings were grown in dark for 12 days in the growth chamber (Percival; Boone, IO) with a light dark cycle of 16h/8h and growth temperature between 20-22°C. Further observations were made using a Leica TCS SP5 confocal scanning head.

2.6 Post-acquisition Image processing

All the images were cropped and processed for their brightness and contrast in Image J (http://rsbweb.nih.gov/ij/) or Adobe photoshop CS3 (www.adobe.com/Photoshop).
Images were assembled using Microsoft powerpoint 2007. For calculating the percent ER membrane in the mutants, a line grid (using grid plugin in ImageJ) was overlayed on every 2D image. Area per point in the grid was set to 30 um\(^2\). Five non-contiguous area points (30 um\(^2\)) per 2D image were chosen to manually calculate the surface area unoccupied by the ER membrane in a given 2D image obtained from individual wt and mutant cells. The unoccupied surface area was subtracted from 30 um\(^2\) to obtain the surface area occupied by the ER membrane for all the 5 grid points analyzed. Data was statistically analyzed using Student’s t-test on a Microsoft excel spreadsheet 2007.

The circularity, perimeter and area of the polygons were also measured by Image J by selecting the area, perimeter and shape descriptors function in the set measurement menu. Circularity is measured by \(4\pi \times \text{area}/\text{perimeter}^2\). A value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape.
Chapter 3: Results

3.1 Creation of tools

Mutants with actin organization defects (crooked, distorted1, distorted2, wurm, klunker, gnarled, arpc4) were used as cell biological tools to study the relationship between actin organization and the endoplasmic reticulum. These actin organization mutants were transformed with three different constructs containing a F-actin binding domain (FABD) of mouse Talin gene fused to a GFP (Kost et al., 1988) to label the F-actin; an ER targeted construct consisting of a red fluorescent protein (RFP) fused to an ER-targeted signal sequence and an ER lumen retention sequence HDEL (Sinclair et al., 2009a); a second ER-membrane construct consisting of Calnexin fused with a fluorescent photoconvertible protein mEosFP (CX::mEosFP) (Mathur et al., 2010). Double transformants of these actin organization mutants were also created to help view both the F-actin and endoplasmic reticulum simultaneously in a cell. Table 3.1 shows the number of lines created.
Table 3.1: Lines created for each of the actin organization mutants and control (Ler) transformed with GFP::FABD2 mTalin, RFP::HDEL and CX::mEosFP

<table>
<thead>
<tr>
<th>Name</th>
<th>Transformed with GFP fused to F-actin binding domain of mouse Talin (GFP::FABD2 mTalin) (Kost et al., 1988)</th>
<th>Transformed with RFP fused to ER lumen retaining signal HDEL (RFP::ER) (Sinclair et al., 2009a)</th>
<th>Transformed with GFP::FABD2 mTalin and Calnexin (CX) fused to fluorescent photoconvertiable protein mEosFP (CX::mEosFP) (Mathur et al., 2010)</th>
<th>Transformed with FABD2:: mTalin and Calnexin (CX) fused to fluorescent photoconvertiable protein mEosFP (CX::mEosFP) (Mathur et al., 2010)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Landsberg erecta</td>
<td>Obtained (Mathur et al., 1999)</td>
<td>Obtained</td>
<td>Created: 15 lines</td>
<td>Created</td>
</tr>
<tr>
<td>crooked</td>
<td>Obtained (Mathur et al., 1999)</td>
<td>Created: 6 lines</td>
<td>Created: 5 lines</td>
<td>Created</td>
</tr>
<tr>
<td>distorted1</td>
<td>Obtained (Mathur et al., 1999)</td>
<td>Created: 1 line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>distorted2</td>
<td>Obtained (Saedler et al., 2004)</td>
<td>Created: 14 lines</td>
<td>Created: 10 lines</td>
<td>Created</td>
</tr>
<tr>
<td>wurm</td>
<td>Obtained (Hulskamp et al., 1994)</td>
<td>Created: 4 lines</td>
<td>Created: 4 lines</td>
<td></td>
</tr>
<tr>
<td>arpc4</td>
<td></td>
<td>Created: 9 lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gnarled</td>
<td>Obtained (Mathur et al., 1999)</td>
<td>Created 7 lines</td>
<td>Created by crossing gnarled GFP::FABD2 mTalin and gnarled RFP::ER</td>
<td>Created</td>
</tr>
<tr>
<td>klunker</td>
<td>Obtained (Mathur et al., 1999)</td>
<td>Created: 9 lines</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2 General morphological observations on the mutants

Mutants with actin organization defects (crooked, distorted1, distorted2, wurm, klunker, gnarled, arpc4) were grown on ½ basal MS media plates. The 7-10 day old seedlings were characterized using a scanning electron microscope (Hitachi TM-1000, Tabletop Microscope). Several organ and cell types like hypocotyl, petiole, cotyledon and trichomes were observed and differentiated between the mutants and wild type (Ler) (Figure 3.1 and Figure 3.2).

All mutants (crooked, distorted1, distorted2, wurm, klunker, gnarled, arpc4) had two phenotypes in common: 1) the presence of distorted trichomes on leaves and stems (Figure 3.2) and 2) the disassociation of neighboring cells to create a scruffy-looking hypocotyl, petiole and cotyledon epidermis (Figure 3.1). Since the trichome cells have already been characterized in some detail with respect to their morphology and F-actin organization (Mathur et al., 1999; Szymanski et al., 1999), epidermal cells of the hypocotyl and petiole were defined as the main focus of this study.

In addition to observing differences in epidermal cell growth, general morphometric measurements of average hypocotyl length, average hypocotyl epidermal cell length and surface area were made. For this wt (Ler background), distorted2 and klunker were grown in the dark, for 7 days and their hypocotyl length from the middle region were measured (Figure 3.3).
Figure 3.1: Scanning electron micrographs of epidermal hypocotyl and petiole cells from 10 day old dark grown seedlings.

(A) Dark grown wild type seedlings show elongated hypocotyl epidermal cells that maintain contact with each other at the cell ends both along the transverse and longitudinal axis.

(B) Dark grown hypocotyl epidermal cells of the *crooked* mutant at the same stage as wt showing elongated cells with gaps created at cell junctions along the long axis (arrow).

(C) Large gaps appearing between neighboring hypocotyl epidermal cells of the *klunker* mutant (arrow), as the cells come apart during hypocotyl expansion and elongation.

(D) Dark grown petiole of the wt showing cells maintaining contact with each other appearing in a well aligned fashion giving a smooth petiole and hypocotyl surface appearance.

(E) Dark grown petiole of the *klunker* mutant depicting scruffiness at the epidermal surface of the petiole as a result of cell detachment and random curling and (F) numerous cells curl out towards the base of the petiole in 10 day old dark grown *klunker*. Scale bar (A), (B)= 10 um, (C),(D),(E),(F)= 100 um
Figure 3.2: Scanning electron micrographs of trichomes from 10 day old light grown wt (A), *crooked* (B) and cotyledon epidermal cells from wt (C), *klunker* (D) seedlings.

(A) Light grown wild type seedling show trichomes in a unicellular, stellate form with 2-4 branches.

(B) Light grown *crooked* seedlings showing trichomes with a random distorted shape.

(C) Light grown cotyledon epidermal cells of wild type seedlings showing a characteristic lobed, jig-saw shape with randomly placed stomata.

(D) Light grown cotyledon pavement cells of *klunker* with cell expansion defects, caused by impaired lobe formation of pavement cells. Large gaps are seen (arrow) in between the pavement cells revealing the underlaying mesophyll layer. Scale bar=100 um

*Arabidopsis* wild type seedlings, grown in the dark for 7 days displayed 1.13±0.10 cm long hypocotyls. However, the mutants *distorted2* and *klunker* at the same stage registered hypocotyl lengths as 0.95±0.16 cm and 0.63±0.06 cm respectively. Using
an unpaired Student’s t-test, significant differences in the hypocotyl length (p<0.05) were noted between the wt (ler) and mutants *distorted2* and *klunker*, indicating a possible effect of the mutation on organ growth and patterning. No significant differences in hypocotyl length were noted between the mutants *distorted2* and *klunker*.

![Average hypocotyl length of 7 day old seedlings](image)

Figure 3.3: Histogram showing the hypocotyl length differences between the wt (ler), *distorted2* (dis2) and *klunker* (klk). The error bars represent standard error. n=10.

Hypocotyl cell surface area (Figure 3.4) and cell length (Figure 3.5) of the epidermal cells present in the middle region of the hypocotyl were further measured in these dark grown 7 day old seedling. In the wildtype epidermal hypocotyls cell length and surface area were 634± 38 um and 15047± 1115 um² respectively. An overall reduction in these parameters was observed in mutants *distorted2* and *klunker* with cell lengths of 293± 19 um and 278± 19 um, and cell surface areas of 8737±565 um² and 6144± 422 um² respectively.
Using an unpaired Student’s t-test significant differences (p< 0.05) were observed between wt and the mutants (distorted2, klunker) in terms of both the cell length and cell surface area. Significant difference in cell surface area were also noted between klunker and distorted2, indicating that klunker has cells of smallest size followed by distorted2 and then wt.

Figure 3.4: Histogram depicting the differences in the average hypocotyl epidermal cell surface area (cells measured from the middle region of the hypocotyl) in dark grown 7 day old seedlings of wt, klunker and distorted. Error bars represent standard error n=15.
Figure 3.5: Histogram depicting the differences in the average hypocotyl epidermal cell length (cells measured from the middle region of the hypocotyl) in the dark grown 7 day old seedlings of wt (ler), distorted2 and klunker. Error bars represent the standard error. n= 13

3.3 General observations of the mutants: F-actin organization

Transgenic wt, klunker, distorted2 and crooked with GFP-FABD-mTalin labeling the F-actin were grown on ½ basal MS media. 7-10 day old seedlings were observed using a Leica DM6000CS microscope equipped with a Leica TCS SP5 confocal scanning head (Leica Microsystems GmbH, Wetzlar, Germany).

As shown in Figure 3.6, thick actin cables were visible in the hypocotyl cells of the mutants of the Arp2/3 complex as seen in distorted2 and crooked. While the actin organization in klunker (mutant of the SCAR/WAVE complex) resembled the fine actin arrangement in Arabidopsis wt.

Based upon the general phenotypic similarity between the different ‘distorted’ class mutants, distorted2 (mutant in ARPC2/p35 subunit of the Arp2/3 complex) and klunker
(mutant in PIR121 subunit of the upstream regulator SCAR/WAVE complex) were chosen for further detailed analysis. These two mutants also had the distinct advantage of showing different F-actin organization.

The following specific objectives were defined:

**Objective 1**- Characterizing ER morphology and dynamics in the *distorted2* and *klunker* mutants.

**Objective 2**- Characterizing ER and F-actin organization in the *distorted2* and *klunker* mutants.

**Objective 3**- Characterizing the effect of different actin organization-disrupting factors like actin interacting drugs, cold treatment, and phosphate starvation on the arrangement and behavior of ER organization in hypocotyl cells of *Arabidopsis*. 
Figure 3.6: F-actin organization in the hypocotyl epidermal cells of 7-10 day old seedlings of wt *Arabidopsis* and *distorted2, klunker, crooked* mutants.

(A) Wild type hypocotyl epidermal cells maintaining contact at the cell ends and showing fine actin arrangement with few longitudinally organized F-actin cables.

(B) Hypocotyl epidermal cells in *klunker* showing very fine/dynamic actin filaments with comparatively few actin cables. Neighboring cells lose contact with each other along the cell axis and big gaps are created (arrow).

(C) Hypocotyl epidermal cells in *distorted2*, curl out of the base of the hypocotyl. Seen is the presence of thick actin cables placed in a random fashion across the curled out cell. Sometimes multiple actin bundles are seen giving a knot-like (star) appearance at the curling tip of the cell.

(D) Hypocotyl epidermal cells in *crooked* showing intense bundling with actin bundles present in an arbitrary arrangement. Also seen is an oddly located stomatal complex (arrow), with individual guard cells being pulled into different directions through their attachment to adjacent cells. Scale bar=10 um.
A

wt

B

klunker

C

distorted2

D

crooked
3.4 Morphological characterization of endoplasmic reticulum in the hypocotyl epidermal cells of *distorted2* and *klunker*

Transgenic lines of *distorted2, klunker, wt* (ler), with endoplasmic reticulum labeled with a fluorescent probe RFP-HDEL (Sinclair et al., 2009a), were used to assess the morphological differences in the cortical and sub-cortical ER. In plants, the cortical ER is present at the cell cortex and is composed of an expanded network of polygons bounded by thin tubules, flattened cisternae, perforated or fenestrated sheets (Sparks et al., 2009) and oval ER bodies (present mainly in plants of the *Brassicaceae*; Matsushima et al., 2003).

Along with the presence of big polygons in the cortical ER of the wt, a few small-sized cisternae and fenestrated sheets were observed. The mutants *distorted2* and *klunker* showed a tremendous increase in the number and size of cisternae and fenestrated sheets (Figure 3.8). Sometimes an entire cell in *klunker* presented huge flattened membranous and fenestrated sheets (Figure 3.7). Percent ER membrane surface area (Figure 3.9) was found to be maximal in *klunker* (80.46±1.85) followed by *distorted2* (64.68±2.93) and then wt (41.36±2.44).
Figure 3.7: Cortical endoplasmic reticulum visualized in the hypocotyl epidermal cells of wt and mutants

(A) Endoplasmic reticulum (ER) in wild type hypocotyl epidermal cells of 12 day old seedlings. The cortical endoplasmic reticulum is an expanded polygon network lined by thin, smooth tubules interspersed with a few flattened sheets of membrane known as cisternae, perforated membrane sheets (fenestrated sheets) and oval ER bodies.

(B) ER in the distorted2 hypocotyl epidermal cells of 12 day old seedlings. In comparison to the wt seedlings at the same stage, the cortical ER in distorted2 was seen in the form of a comparatively small-sized polygon network lined by convoluted tubules and an increased number and size of flattened membrane sheets (cisternae), fenestrated sheets and oval ER bodies.

(C) ER in klunker hypocotyl epidermal cells of 12 day old seedlings. Sometimes, a major cell portion was covered by expanded ER membrane sheets (cisternae) along with abnormal looking ER bodies.

(D) ER in the klunker hypocotyl epidermal cells of 12 day old seedlings. In comparison to wt seedlings at the same stage, klunker showed highly expanded ER membrane sheets (cisternae), fenestrated sheets and oval ER bodies with a scattered distribution of ER polygons lined by convoluted tubules. Scale bar=5 um.
Figure 3.8: Diagrammatic representation depicting the differences in the endoplasmic reticulum between the wt (ler) and mutants (distorted2, klunker)
Figure 3.9: Histogram depicting the variation in the percent surface area covered by ER membrane in wt and distorted2(dis2) and klunker(klk). Error bars represent the standard error. Average columns and standard deviation are based on the percent surface area covered by the ER membrane from five separate regions (30 um²) in 25 images obtained from 25 individual cells each of wt, distorted2 and klunker.

Various parameters like polygon area, polygon boundary length (perimeter), circularity, ER membrane density, ER body number and ER body surface area were used to show the morphological differences in the cortical ER between wt, klunker and distorted2. The cortical ER in wt was present in the form of widely extended polygonal networks bounded by thin tubules (Figure 3.10) with an average polygon area 12.24±2.04 um² and polygon boundary length of 15.11±1.34 um. Using unpaired Student’s t-test (p<0.05), mutants distorted2 and klunker were shown to have significant reduction in polygon size with surface area of 2.29±0.41, 2.34±0.36 um² and boundary length of 5.56±0.51, 6.06±0.50 um respectively.
The wt polygons appeared in the form of an expanded network lined by smooth, thin tubules. The mutants *distorted2* and *klunker*, however, displayed loosely packed small polygons, surrounded by convoluted tubules (Figure 3.10). This surface property of tubules lining the ER polygons, appearing as smooth vs. convoluted between the wt and mutant was measured by comparing the circularity of polygons. The idea behind this was that smooth tubules would be better at creating a polygon shape as observed in the wt, while a convoluted tubule would make the polygon look circular. Circularity of these polygons were measured using Image J, where a value of 1 indicated a circle and as the value approached 0, it indicates an increasingly elongated shape. Unpaired Student’s *t*-test was used to indicate significant differences in the polygon circularity between the mutants (*klunker* and *distorted2*) and wt.

![Morphological differences in ER polygons of 12 day old seedlings](image)

Figure 3.10: Histogram showing differences in the ER polygons between wt and mutants (*distorted2* (*dis2*), *klunker* (*klk*)) in terms of polygon surface area, perimeter and circularity. Columns show average values and error bars show standard error based on measurements taken from 60 polygons for each of the wt, *dis2* and *klk* from 15 images of 15 individual wt, *dis2* and *klk* cells.
Unpaired Student’s t-test was used to show significant differences (p<0.05) in the number of ER bodies (Figure 3.11) between the wt (3.9±0.48), distorted2 (6.5±0.96) and, klunker (10.5±1.60). Usually the ER bodies in mutants were present in the form of an oval but sometimes appeared in the form of abnormally elongated structures clinging to each other and forming either linear or circular strings (Figure 3.7)

![ER body number & surface area in 12 day old seedlings](image)

Figure 3.11: Histogram revealing deviations in the ER body number and area between wt and distorted2 and klunker. Columns represent the average number of ER bodies and surface area with error bars showing the standard error based on the number and size of ER bodies in 15 images(obtained at the same microscope settings mentioned in material and method section) from 15 individual wt, dis2 and klk cells.
3.5 Understanding the morphological dependence of the endoplasmic reticulum on the F-actin organization between the wt and mutant’s \textit{distorted2} and \textit{klunker}.

Double transgenic lines transformed with a red fluorescent protein linked to ER lumen retaining signal sequence (Sinclair et al., 2009a) and a green fluorescent protein fused to the F-actin binding domain of mammalian Talin gene (Kost et al., 1988) were used to label and visualize actin and ER simultaneously in the epidermal hypocotyl cells of the wt, mutant of the Arp2/3 complex \textit{distorted2} and its upstream regulators \textit{klunker}.

These transgenic seedlings labeling the actin and ER in wt, \textit{distorted2} and \textit{klunker} were used to differentiate and develop a correlation between the actin and ER arrangement. As seen in Figure 3.12 and Figure 3.13, wt hypocotyl epidermal cells displayed fine actin filaments with a few actin strands running longitudinally across the cell. Well defined F-actin arrangement in wt produced expanded ER polygons lined by smooth tubules and a few fenestrated sheets and cisternae. In comparison, \textit{distorted2} cells showed extensively bundled F-actin either placed randomly or running longitudinally across the cell. The ER morphology in the hypocotyl cells of \textit{distorted2} varied according to the actin configuration and arrangement present in the cell. When actin is in the form of thick bundles lying close to each other and running longitudinally across the cell, ER membrane appears as small-sized, polygons lined by convoluted tubules with a few fenestrated sheets and cisternae. However, when actin bundles, arrange themselves in a random fashion, ER is present as flat membranes and fenestrated sheets (Figure 3.12). These different forms of ER and actin were also visible within the same \textit{distorted2}
hypocotyl epidermal cells, where one region of the cell with highly bundled and randomly placed actin had ER in the form of flattened sheets, while the other region of the same cell with comparatively sparse actin bundles formed ER polygons with a few small flattened sheets (Figure 3.14). The actin configuration in a cell was seen to be precisely followed by the ER membrane arrangement. In areas where no evident actin bundles or fine actin were present, ER membranes were also visibly absent (Figure 3.15).

The mutant of the SCAR/WAVE complex, *klunker*, depicts a very fine F-actin organization with few or no actin bundles observed. ER in *klunker* arranges accordingly in the form of huge flattened sheets and membranes.
Figure 3.12: Simultaneous visualization of F-actin and ER in the hypocotyl epidermal cells of wt, *distorted2* and *klunker*

(A) Actin, (B) ER and (C) merged image of hypocotyl epidermal cells of the wt. Displayed is a fine arrangement of F-actin with a few F-actin bundles overlaid by a highly expanded polygonal reticulate ER network made of huge polygons surrounded by smooth tubules and a few cisternae and fenestrated sheets.

(D) Actin (E) ER and (F) a merged image of the hypocotyl epidermal cells of *distorted2*. Shown above is an increased number of highly bundled actin strands running longitudinally across the cell. Overlaying this is an ER network composed of small sized polygons surrounded by convoluted tubules and a few flattened ER membrane sheets.

(G) Actin, (H) ER and (I) a merged image of the hypocotyl epidermal cells of *distorted2*. Demonstrated are highly bundled, randomly placed actin strands, overlaid by an ER network mostly composed of flattened membrane and fenestrated sheets.

(J) Actin, (K) ER and (L) a merged image of the hypocotyl epidermal cells of *klunker*. Actin arrangement is seen in the form of a diffuse fine actin network with either few or no actin strands. ER network is seen as fenestrated sheets and cisternae. Scale bar=5 um
Figure 3.13: Diagrammatical representation of actin and ER arrangement in the epidermal hypocotyls cells of wt and mutants distorted2 and klunker based on actual observation shown in Figure 3.12

Figure 3.14: Simultaneous visualization of F-actin and ER in the hypocotyl epidermal cells of the distorted2

(A) Actin, (B) ER and (C) merged image of the hypocotyl epidermal cells of distorted2. Depicted in this image are two different actin and ER arrangements within the same cell. In one case (circle) when there is excessive actin bundling present close enough and randomly, ER appears in the form of flattened membrane sheets (cisternae). When there are comparatively few actin bundles (rectangle), ER is present in a polygonal form with a few flattened sheets interspersed in between.
Figure 3.15: Simultaneous visualization of F-actin and ER in the hypocotyl epidermal cells of distorted2

(A) Actin, (B) ER and (C) merged image of the hypocotyl epidermal cells of distorted2. The image depicts a precise correlation between the F-actin organization and the ER arrangement. F-actin in distorted2 forms thick bundles lying closely in a random fashion. ER membrane appears to follow the tracks laid by the F-actin creating a network of loosely packed small polygons, fenestrated sheets and cisternae. Areas of the cell that do not have F-actin tracks do not have indications of presence of the ER membrane (star). Scale Bar=5 um
3.6 Observing the effect of several factors known to influence the actin organization in a plant cell, on the configuration and behavior of the ER. These include actin interacting drugs, cold stress and phosphate starvation.

Latrunculin-B treatment affecting the endoplasmic reticulum in wt Arabidopsis hypocotyl cells

Transgenic Arabidopsis wt (Ler-background) seedlings transformed with red fluorescent protein fused with the ER lumen retaining sequence HDEL (Sinclair et al., 2009a) were grown on ½ basal MS medium. Observations were made on dark grown 12 day old wt seedlings treated with 500 nm of Latrunculin-B or with water (control) for 1hr, using a confocal microscope.

As shown in Figure 3.16, the ER in the control seedlings left in water for an hour was in the form of meandering, loosely formed, small ER polygons with a few flattened ER membranes appearing randomly in the network. These were unlike the expected morphology observed in wt 12 day old Arabidopsis seedlings observed directly from the solid, agar medium plates, which possess a typical spread of smoothly lined ER polygons. The latrunculin-B treated seedlings were characterized by the presence of comparatively big polygons, arbitrarily present small-sized fenestrated sheets and randomly distributed, huge ER lamellar sheets (cisternae) across the cell.
Figure 3.16: Endoplasmic reticulum in the hypocotyl epidermal cells of the 12 day old seedlings in (A) control (wt seedlings in water) (B) Latrunculin-B treated wt seedlings.

(A) Endoplasmic reticulum in the hypocotyl epidermal cells of 12 day old seedlings left in water (control) for an hour. The ER polygons appear loosely packed surrounded by convoluted tubules. A few small-sized cisternae also appear in between an expanded polygons network.

(B) Endoplasmic reticulum in the hypocotyl epidermal cells of 12 day old seedlings left in 500 nm Latrunculin-B (in water) for an hour. Along with the presence of ER polygons made by smooth tubules, a few small-sized fenestrated sheets present at arbitrary places and huge lamellar patches of ER membrane were seen randomly distributed along the ER reticulate network. Scale bar=5um

 prolet Cisternae ER-polygon

Differences between the water and Lat-B treated seedlings were found to be statistically significant using an unpaired Student’s t-test. The average cisternae surface size (Figure 3.18) in latrunculin-B treated seedlings was 51.90±8.45 um² compared to 7.30±1.02 um² in the control. However, the ER polygons (Figure 3.17) in the Lat-B treated seedlings had a significantly higher surface area (area: 12.70± 1.51 um²) than the control (area: 8.32±0.76 um²).
Figure 3.17: Graph displaying morphological differences in the ER polygons of Latrunculin-B treated wt Arabidopsis seedlings in terms of polygon surface area, perimeter and circularity. Columns show average values and error bars show standard error based on measurements taken from 40 polygons for each of the control and Lat-B treated seedlings from 20 images of 20 individual control and Lat-B treated cells.
Figure 3.18: Graph displaying differences in the average individual cisternae surface area of Latrunculin-B treated and control wt Arabidopsis seedlings. Error bars represent the standard error. Measurements are based on n= 47;control and n=39;Lat-B treated individual cisternae surface area measurements taken from 20 images of 20 individual control and Lat-B treated cells.
The effect of cold stress on the ER in hypocotyl cells of wt Arabidopsis seedlings

Transgenic Arabidopsis wt seedlings carrying a red fluorescent protein fused to ER lumen retaining signal sequence HDEL (Sinclair et al., 2009a) helping to visualize the ER were grown on ½ basal MS medium. Observations were made on dark grown 12 day old seedlings left at 4°C for 24 hours and normal growth temperature (23°C) as a control, using Leica DM6000CS microscope equipped with a Leica TCS SP5 confocal scanning head (Leica Microsystems GmbH, Wetzlar, Germany). Statistical analysis was performed using unpaired Student’s t-test.

As shown in Figure 3.19, 3.20 and 3.21, ER under normal growth conditions of 23°C had a characteristic appearance of highly expanded smoothly lined polygons (area: 21.02± 1.84 um², perimeter:19.53±1.06 um, circularity 0.68±0.02) with a low percent area covered by the ER membrane (41.78± 1.70%). However, seedlings subjected to chilling conditions for 24 hrs, showed significant phenotypic reduction in terms of the polygon area (7.10±0.69 um²) and perimeter (10.71±0.58 um). The polygons were lined by convoluted tubules and appeared rounded (roundness: 0.74± 0.10), with a majority of cortical ER composed of lamellar sheets (percent surface area covered by ER membrane 64.5± 2.3%).
Figure 3.19: Endoplasmic reticulum in the hypocotyl epidermal cells of 12 day old seedlings kept at (A) normal growth temperature (23°C) (B) 4°C for 24 hrs

(A) ER in the control hypocotyl epidermal cells of 12 day old seedlings kept under normal growth temperature (23°C). As observed, the ER is present in the form of highly expanded sheets of polygons lined by thin smooth tubules with a random distribution of few small sized cisternae.

(B) Endoplasmic reticulum in the hypocotyl epidermal cells of 12 day old seedlings kept in 4°C for 24 hrs. ER appeared in the form of loose flowing polygon sheets surrounded by convoluted tubules and randomly present small sized cisternae. Scale bar=5 um
Figure 3.20: Graph displaying morphological differences in the ER polygons under normal and cold stress conditions wt seedlings, in terms of polygon surface area, perimeter and circularity. Columns show average values and error bars show standard error based on measurements taken from n=37; control and cold stressed polygons for each of the control and cold stress treatments from 20 images of 20 individual control and cols stress treated cells.
Figure 3.21: Graph displaying percent surface area of the ER membrane under normal and cold stress conditions. Error bars represent the standard error. Average percent surface area occupied by the ER membrane and standard deviation are based on the percent surface area occupied by the ER membrane within a cell, from five separate regions (30 \( \mu \text{m}^2 \)) in 20 images obtained from 20 individual control and cold stress treated seedlings.
The effect of phosphate starvation and increase on the ER in the hypocotyl cells of wt Arabidopsis seedlings

Many actin regulating proteins are known to be themselves modulated by the ionic conditions in the environment. Altering the ionic environment may disturb the actin regulatory process bringing about a consequent change in the actin organization and behavior in a cell.

Transgenic Arabidopsis wt seedlings transformed with a red fluorescent protein fused to ER lumen retaining signal sequence HDEL (Sinclair et al., 2009a) were grown on basal MS media with three different phosphate concentrations; 170 mg/l, 340mg/l and under phosphate starvation condition. Observations were made on dark grown 12 day old seedlings, using Leica DM6000CS microscope equipped with a Leica TCS SP5 confocal scanning head (Leica Microsystems GmbH, Wetzlar, Germany).

The phosphate starved wt Arabidopsis seedlings appear stunted with reduced hypocotyl and root length (Figure 3.22). At the subcellular level, as observed in Figure 3.23, 3.24, 3.25, polygon size and circularity was affected by the phosphate ion concentration. When Arabidopsis wt seedling were grown on 170 mg/l of phosphate concentration, the polygons were the largest lined and were lined by straight tubules (area: 21.19±1.85 um²; perimeter: 20.87± 1.11 um, roundness: 0.61±0.02) followed by 340 mg/l of phosphate concentration (area: 18.17±2.13 um², perimeter: 18.08± 1.22 um, roundness: 0.65± 0.02) and then phosphate starvation (area: 8.43± 1.13 um², perimeter: 12.06± 0.81 um, roundness: 0.67±0.02) conditions. Correspondingly, percent ER membrane was lowest under 170 mg/l of phosphate concentration (34.25±2.1%),
proceeded by 340 mg/l of phosphate concentration (45.65±3.4%) and then phosphate starvation (56.54±3%). The phosphate starvation conditions therefore affected the ER morphology and significantly affected the polygon and cisternae size and distribution.
Figure 3.22: *Arabidopsis* wt seedlings grown on MS media with different phosphate concentrations.

(A) When grown on 170mg/l of phosphate, the dark grown seedlings show a hypocotyl etiolation response and normal cotyledon and root development.

(B) Under no phosphate conditions, germination rate is affected and the dark grown seedlings show a stunted appearance with reduced hypocotyl and root length.

(C) Under 340mg/l of phosphate, the dark grown seedlings look similar to the normal phosphate conditions with elongated hypocotyl and normal root development.
Figure 3.23: Endoplasmic Reticulum in the hypocotyl epidermal cells of 12 day old seedlings grown on MS media with different phosphate concentrations.

(A) Endoplasmic reticulum in the hypocotyl epidermal cells of 12 day old seedlings grown on 170 mg/l of phosphate concentration. ER observed is present in a highly expanded reticulate form composed of smoothly lined tubules and sparsely present small flattened sheets or cisternae.

(B) Endoplasmic reticulum in the hypocotyl epidermal cells of 12 day old seedlings grown on 340 mg/l of phosphate concentration. ER observed looks comparatively dense composed of small sized polygons lined by smooth tubules. Also observed is a comparative increase in the randomly formed fenestrated sheets and cisternae.

(C) Endoplasmic reticulum in the hypocotyl cells of 12 day old seedlings on phosphate starvation. ER observed is in the form of a dense and a flowing polygon network composed of rounded polygons lined by convoluted tubules and increased number and size of fenestrated sheets and cisternae. Scale bar=5 um.
Figure 3.24: Graph showing morphological differences in the ER polygons wt seedlings are grown on MS media with different phosphate concentrations. Columns show average values and error bars show standard error based on measurements taken from n=35; 170 mg/l of phosphate, n=30; phosphate starvation and, n=50; 340 mg/l of phosphate for each phosphate treatment from 20 images of 20 individual cells.
Figure 3.25: Graph showing average percent surface area occupied by the ER membrane under phosphate stress conditions. Error bars represent the standard error. Values are based upon the percent surface area occupied by the ER membrane within a cell, from five separate regions (30um²) in 15 images obtained from 15 individual cells each.
Chapter 4: Discussion

4.1 Actin organization dependent change in ER morphology of *distorted2* and *klunker*

Actin dependent remodeling of the ER network, consisting of cisternae and interconnected tubules, is well known in plants. The correlations have been established mainly through the effects that different actin disrupting drugs have on ER appearance and behaviour (Liebe et al., 1995; Sparks et al., 2009; Staehelin, 1997). Actin disrupting drugs like latrunculin-B (Lat-B) act by binding to globular actin monomers and prevent F-actin polymerization and further assembly (Morton et al., 2000). Similarly, Cytochalasin-D, another commonly used inhibitor, binds to actin filaments and prevents polymerization (Schliwa, 1982). Due to their mechanism of action, these drugs could also indirectly limit access to and interactions between different actin regulatory proteins such as profilins and actin depolymerizing factors (ADFs) as well as nucleating and branching complexes such as the Arp2/3 complex and existing actin filaments. Whereas a recent model suggests the Arp2/3 complex acts as a major player in organizing the ER (Sparks et al., 2009), to date, there is no experimental evidence for this link. At the same time, the Arp2/3 complex and its proposed behavior in actin organization make it an attractive candidate for this purpose.

The Arp2/3 complex is directly implicated in creating new actin filament branches from the sides of existing actin filaments at a precise angle of 70° (Mullins et al., 1998) and thus produces a dendritic actin array (Svitkina and Borisy, 1999). A very similar dendritic array is apparent in the makeup of open-ended polygons created by
membrane tubules of the cortical ER. The morphological similarity between dendritic F-actin organization and the cortical ER raises the possibility that the Arp2/3 complex plays a direct role in organizing the form of the ER. The present study was aimed at exploring this possibility. The investigation relied on simultaneous visualization of the ER and actin organization in several Arabidopsis lines having mutations in different subunits of the Arp2/3 complex and its upstream regulatory SCAR/WAVE complex.

Arabidopsis thaliana plants having mutations in Arp2/3 as well as the SCAR/WAVE complex subunits exhibit similar morphological defects (Mathur, 2005). The most recognizable and common mutant phenotype consists of randomly distorted trichomes, while epidermal cells of the petioles and hypocotyl as well as the jigsaw shaped cotyledon pavement cells also exhibit a misshapen look. Mutant epidermal hypocotyl cells, when forced to elongate rapidly under low light conditions, loose contact with their neighboring cells and curl out of the base of the hypocotyl. In addition, gaps start appearing between neighboring cells of the hypocotyl, petiole and cotyledon pavement cells. It has been speculated that the intercellular gaps are formed as a result of varied and non-synchronized elongation rates of the neighboring cells in the mutants. Consistent with this view of disturbed cell elongation rates in the ‘distorted’ class of mutants, significant differences in the hypocotyl length, epidermal cell length and epidermal surface area were noted between wild type and mutants (distorted2, klunker). Studies have also reported an overall reduction in plant size in wurm and decrease in fresh weight in distorted1 and distorted2 mutants (Le et al., 2003; El-Assal et al., 2004). The change in the elongation and expansion rate of the mutant cells suggested a possible role played by the Arp2/3 complex in active cell growth where the intracellular...
trafficking of vesicles and sub-cellular components is controlled by actin cytoskeleton dynamics (Mathur, 2005).

Indeed, actin organization in these mutants is abnormal compared to wild type Arabidopsis and is most apparent in cells such as leaf epidermal trichomes, elongating hypocotyl and petiole cells and root hairs undergoing active growth during morphogenesis (Mathur et al., 2003a,b). Thick bundles of F-actin have been observed in the hypocotyl cells of the distorted1, distorted2, crooked, wurm, arpc4 mutants as compared to a rather diffuse F-actin network seen in hypocotyl cells of Arabidopsis wt. In comparison, the SCAR/WAVE mutants such as klunker and gnarled display fine F-actin configuration, similar to what is observed in the hypocotyl epidermal cell of the wt (Mathur et al.,2003a; Mathur et al.,1999; El-Assal et al.,2004;Li et al.,2003). Trichomes in the Arp2/3 complex mutants have accumulate of thick aggregates/ bundles of actin in small randomly located pockets across the cell (Mathur et al.,2003a, Mathur et al., 2003b, Li et al.,2003).

Interestingly, actin drug treatments also create the same phenotypic and sub-cellular effects such as the formation of distorted trichomes containing ‘short, thick rods or bundles’ of F-actin (Mathur et al., 1999; Szymanski et al., 1999). Following observations of organelle aggregates and low velocity of movement of Golgi bodies and peroxisomes in crooked trichomes (Mathur et al., 2003), it has been suggested that areas with high actin bundling have decreased cell expansion due to low rates of vesicle motility and delivery, whereas areas with diffuse F-actin expand normally, apparently due to the un-interrupted vesicle trafficking (Mathur et al., 2005).
Early investigations confirmed the above observations in different mutants. Thereupon, having noted the similarity in phenotypes, two mutants were selected for further analysis of the correlation between the actin cytoskeleton and the endoplasmic reticulum. These were *distorted2* (mutant in ARPC2 subunit of the Arp2/3 complex) and *klunker* (mutant in the PIR121 subunit of the upstream SCAR/WAVE regulator complex of the Arp2/3 complex). The mutants *distorted2* and *klunker* were transformed with fluorescent probes targeted to the actin or the ER targeting to view and study the behavior of the F-actin and ER.

There were notable differences in F-actin organization between *distorted2* and *klunker* suggesting that there might be differences in their ER organization too. The ER in higher plants is made up of two major domains; cortical ER and subcortical ER. The cortical ER is further composed of an interconnected polygon tubular network, cisternae (flattened ER membranes), perforated sheets (fenestrated sheets) and oval shaped ER bodies. The cytoplasmic ER on the other hand, depicts fast flowing cytoplasmic/transvacuolar ER strands supported by the actin cytoskeleton (Sparks et al., 2009; Staehelin, 1997). Differences in these individual domains of the ER were distinct between wt, *distorted2* and *klunker* when viewed in mature expanded epidermal cells of etiolated hypocotyls. In the mature elongated wt hypocotyl cell, actin exists in the form of a few F-actin cables stretched along the longitudinal cell axis along with the fine cortical actin meshwork. In these cells, the ER is present in an extensively spread polygon network lined with thin, smooth tubules, and sparse cisternae and fenestrated sheets. A similar ER morphology has also been reported by Ridge et al., (1999) in the mature wt *Arabidopsis* hypocotyl cells where ER is described as predominantly reticulate. The ER
membrane in wt hypocotyl cells is thought to be shaped into ‘big’ and ‘smoothly lined’ polygons by expansion across the sparsely present longitudinally running actin strands.

In distorted2 F-actin is present in either increased number of thick actin bundles adhering closely to each other along the longitudinal axis or as close, randomly placed, loose actin cables. Under circumstances where actin is seen in a number of thick strands closely stretched across the cell, the ER membrane extends along these closely placed bundles producing small loose polygons. The expansion of ER membrane is not as much as visible in the wt because the actin cables are lying very close. In the wt, the ER membrane can expand to produce big polygons lined by smooth thin tubules along the far apart, stretched actin cables. When actin is present in the form of thick, randomly placed actin cables, the flattened ER membrane is not able to expand between these arbitrarily placed actin bundles. The result is the formation of either fenestrated sheets or large cisternae. By contrast the mutant of the SCAR/WAVE complex, klunker shows a very fine F-actin organization. In klunker, the flattened ER membranes remain intact with fewer numbers of polygons. The difference might be due to the lack of pulling force attributed to the absence of F-actin cables. Absence of actin cables in the mutant, klunker also pointed towards a possible interference in the activity of myosin motor complexes, which are involved in the pulling of the ER membrane by associating with the actin cables, creating ER tubules (Yokota et al., 2011)

The polymerized form of actin in a normal, living, plant cell can exist in three morphologically distinguishable and readily interchangeable states: 1) a fine meshwork comprising of dynamic actin filaments, 2) thick F-actin bundles and 3) loose F-actin
strands (Baluska and Mancuso, 2009). These three forms of polymerized actin are created and maintained by several actin interacting proteins, working in a balanced state within a plant cell. Under normal conditions the Arp2/3, complex is functional and a dynamic actin nucleation and polymerization process results in the creation of fine dendritic actin arrays. Under conditions where the Arp2/3 complex is mutated, the proportion of these three actin forms is apparently disturbed because of an imbalance created among the actin interacting proteins which are involved in creating and maintaining the polymerized actin filament network.

The mutants *distorted2* and *klunker* are supposed to lack the formation of dendritic actin array by the Arp2/3 complex formed under normal conditions. As has been visible using mutant transgenic lines transformed with GFP targeted to F-actin, *distorted2* shows intense actin bundles and a low frequency of a fine actin meshwork/dendritic actin array due to the non-functionality of the Arp2/3 complex. On the other hand *klunker* shows a highly dynamic form of actin due to the disruption in the upstream regulator the Arp2/3 complex. Since the actin is supposed to be in a very dynamic state in *klunker*, a fine actin filament array is created with few actin cables.

Cortical ER in a living plant cell is present in three major forms: 1) flattened ER membrane sheets/ cisternae, 2) thin tubules forming the ER/polygons and 3) ER membrane sheets with perforations called fenestrated sheets. It is suspected that the interchangeable behavior of these forms is highly influenced by the dynamic dendritic actin array in accordance with the organization of the three described forms of actin.
The variation in the actin forms created within a cell influences the ER morphology and dynamics. Depending on the orientation and configuration of the F-actin bundles, either ER tubule formation and maintenance or the creation of fenestrated sheets could result. In the mutants, the different patterns of scaffold formed by the F-actin is changed which indirectly influences the various patterning of ER forms. When the dynamic dendritic array is limited and thick cables of actin lie close to each other in distorted2, the ER membranes do not have the chance to extend and create fine tubules along the dendritic tracks layed down by the activity of Arp2/3 complex. In this case, the membrane exists in the form of loose polygons sheet with convoluted tubules or membrane sheets with perforations or just flat lamellar sheets. In klunker, F-actin exists in a very dynamic form with very few or no bundles forming and the ER membranes do not have an underlying actin support and the pulling force normally generated by the actomyosin system is lacking. The ER membranes are therefore maximally present in the form of cisternae unlike the highly expanded polygonal network seen in wt.

The F-actin and ER relationship described above may be likened to a wire with a loop at one end that is used to create soap bubbles. The long wire resembles an actin cable and the soap bubble that gets formed at the looped end resembles an ER membrane sheet/ cisternae. In this scenario, regulating the size of the loop allows a change in the size of the bubble. Thus, pulling on the actin (wire) can create changes in the size of ER polygons. Increased pull and opening out of the wire loop causes holes to appear in the soap film and resembles the fenestrated ER. Further opening of the loop causes the bubble to collapse and wrap around the wire to provide a likening to the tubules making up the polygonal form of the endoplasmic reticulum.
The mutants \textit{(distorted2 and klunker)} also displayed increased number of abnormally elongated ER bodies, sometimes surrounding the perinuclear region. These abnormal looking ER bodies have also been reported in myosin XI-K double and triple knockout mutants (Ueda et al., 2009). This suggested the involvement of actomyosin system in the ER body shape development. Induction of ER body formation during wound stress in \textit{Arabidopsis} have suggested a role in creating a defense mechanism against herbivores (Matsushima et al., 2003). This increase in the number of ER bodies in the mutants \textit{(distorted2 and klunker)} is speculated to occur either because of the cell-cell detachment and peeling of epidermal cells in the mutant, stimulating a wounding response or somehow is an indirect effect on myosin mobility due to abberent actin configuration in the mutants.

\textbf{4.2 Factors disrupting the actin organization also influence shape and behaviour of ER in wt \textit{Arabidopsis} hypocotyl cells}

Observations on plants having mutations in different subunits of the Arp2/3 complex and its upstream regulator \textit{(distorted2, klunker)} show clear morphological differences in the endoplasmic reticulum. Similar features of the ER existing as “large lamellar sheets” (Liebe et al., 1995), “ER accumulation in the form of patches” (Knelbel et al., 1999), and “increased area and size of cisternae” (Sparks et al., 2011) have been reported by studies commonly using actin disrupting drugs. In a model proposed in a study done by Sparks et al. (2009), it was suggested that in the presence of actin disrupting drugs, the actomyosin system may become unstable resulting in the formation of cisternae/flattened lamellar sheets, similar to that observed in the present study. Furthermore, another study done by
Ueda et al. (2010) reported ‘large ER aggregates’ in multiple myosin knockout mutants also hinting towards a possible role of myosins in shaping the ER.

To verify these observations and authenticate the ability of these drugs to reproduce the phenotype of ER reported in Arp2/3 mutants by this study, the ER behavior in the *Arabidopsis* wt seedling was observed after the application of Latrunculin-B. Latrunculin-B prevents the process of actin polymerization, by sequestering G-actin monomer (Morton et al., 2000). As observed in Figure 3.16, the *Arabidopsis* wt seedlings after being treated with 500 nmol of Latrunculin-B for 1 hr produced random patches of huge ER membrane sheets. These lamellar sheets might form due to the absence of any actin scaffolds to establish the ER morphology, also previously observed in the *klunker* mutant. Other ER morphologies like the formation of large fenestrated sheets and small sized polygons visible in mutant were not observed in wt treated with Lat-B. These differences in ER morphologies between the mutants and the wt treated with Lat-B might be because the actin polymerization process is completely halted when wt is treated with the actin disrupting drugs like Lat-B, however, in the mutants, the polymerized form of actin still exists even though an imbalance appears in the three polymerized forms of actin; 1) a fine meshwork comprising of dynamic actin filaments, 2) thick F-actin bundles and 3) loose F-actin strands existing in the cell.

Another noticeable feature in these Lat-B-treated seedlings is the presence of large polygons lined by thin, smooth tubules visible of the same size as seen in the 12 day old wt *Arabidopsis* seedlings. The polygons in Lat-B-treated wt seedlings were significantly larger than the ones in control (seedlings in water for an hour). The large and static
polygons in the seedlings treated with Lat-B, is because of the nonfunctional actomyosin system which may act together in the creation and maintenance of polygons. As there is no polymerized actin formed in the wt cells treated with the actin disrupting drugs, there are no tracks on which the motor proteins can run and shape the ER polygons and this the polygons appear static compared to the ever-changing and dynamic ER polygons network in control. The small polygon size in the control (seedlings in water for 1 hr) may be due to hypoxic conditions. Under hypoxic conditions, the ATP production is affected in a cell which further can indirectly affect the ATP-dependent movement of myosin motors on the actin filaments. The inability of motor molecules to track along the actin filaments would further create a lack of pulling force, required to pull the membrane along the actin tracks shaping the membrane into thin tubules surrounding large polygons.

Therefore another aspect explored by this study was the effect of phosphate starvation or excess availability, on the ER structure and behavior. Phosphate ions form a part of the ionic pool involved in the modulation activity of several actin interacting proteins. Organic phosphorus is also bound to phospholipids, forming a major component of eukaryotic cell membrane. The double mutant pah1/pah2 is reported to have an overproduction of phospholipids, causing overexpansion of ER membrane forming large lamellar sheets (Eastmond et al., 2010). As observed in figure 3.23, significant differences were noted between the ER polygon size and percent ER membrane coverage between the treatments. Arabidopsis wt seedlings grown on a phosphate concentration of 170 mg/l resulted in cells having the largest polygons lined by thin smooth tubules followed by wt seedling grown on 340 mg/l of phosphate concentration and seedling grown under phosphate starvation conditions. The percent surface area within a cell
occupied by the ER membrane was highest under phosphate starvation conditions indicating a higher occurrence of a loosely packed polygons lined by convoluted tubules, fenestrated sheets and cisternae. Phosphate is required for the production of ATP within a cell and the movement of motor proteins on the actin filaments is also ATP dependent. Under phosphate starvation conditions, all the available phosphate reserves within a cell are utilized for maintenance and repair for the cell to survive and other processes requiring phosphate like ATP dependent myosin movement on the actin filaments may be shut down or slowed. Phosphate overproduction could create potentially toxic conditions similar to the cells treated with ROS. Thus, both phosphate starvation and phosphate over-production tend to effect the actomyosin system in a cell further influencing the shape and organization of ER.

Other abiotic factors like chilling temperatures were also tested as a part of the study to affect the creation of an extended ER sheet. *Arabidopsis* wt when left under cold stress conditions for 24 hr developed ER in the form of loose ER polygons. The polygon shape and size in cold stressed seedlings were found to be significantly different from the seedlings left under normal growth conditions. A similar reorganization of endoplasmic reticulum was observed in the epidermal cells of onion bulb scales after cold stress (Quader et al., 1988), agreeing with the present observations. The study speculated a change in the force generating actomyosin system at low temperatures (Quader et al., 1988). The physical properties of cell membranes, lipid-lipid and lipid-protein interaction is temperature dependent. At low temperatures, there is a decrease in cell’s fluidity and permeability. The decrease in cell’s permeability further affects the movement of vital molecules and ions across the cell membrane. The altered ionic
environment in a cell can possibly affect the activity of various actin interacting proteins shaping the actin cytoskeleton. In addition, cold temperature could cause cellular reaction to slow down or even stop like the movement of myosin motors on actin filaments. The slowed or halted myosin movement could directly cause an effect on the ER morphology and behaviour under cold conditions.

4.3 Conclusion

This study aimed to investigate the relationship between F-actin organization and the endoplasmic reticulum. It was hypothesized that a major regulator/key regulator, the Arp2/3 complex, would have a direct effect on the way ER polygons and cisternae are organized through its fashioning of the dendritic F-actin array. *Arabidopsis thaliana* (*Landsberg erecta*) was chosen as the wild type control for general observations on mutants in different subunits of the Arp2/3 complex and its regulator SCAR/WAVE complex. The major findings are:

The relative organization of ER into cisternae, fenestrated sheets and polygons made up of ER tubules is dictated by the underlying actin organization. Actin bundles and cables are required for the expansion of ER membranes and inter-bundle distance might create either fenestrated sheets or allow the collapse of membranes around/on F-actin to form polygons. An inability to form F-actin bundles due to increased actin dynamics or inability of actin to polymerize results in large patches of the ER membrane observed as cisternae. In the absence of experiments that directly show an Arp2/3 complex interacting with F-actin to reorganize the ER we are limited to the use of mutants in different subunits of the complex. A general assumption based on the common
phenotype of the mutants belonging to the Arp2/3 complex and its upstream regulators, suggests, that the complex is not fully functional in these plants. Thus our observations strongly suggest a direct link between the complex activity and ER organization. They also provide the baseline work for carrying out detailed in vitro reconstitution assays. In addition, the work highlights the fact that changes in localized ionic conditions, alterations in temperature, pH and other abiotic factors that in turn might affect the activity of the complex have a pivotal role in organizing the ER.

4.4 Future prospects

The study opens up and leaves several unanswered questions and concerns for future investigations. It would be important to know if the affected ER morphology in the Arp2/3 mutants also consequently influences its dynamic behaviour. The study discusses the ER morphology affected in the ‘distorted’ mutants klunker and distorted2, however, the ER behaviour in the other distorted mutants (distorted1, crooked, wurm, gnarled, arpc4) still needs to be explored in detail. The observations made on the epidermal hypocotyl cells of etiolated Arabidopsis, should also be extended to other cell types. Further on, it would be important to study the effect of aberrant ER morphology in the Arp2/3 mutants, on the other domains of the endomembrane system like Golgi bodies. The ER morphology and behaviour is seen to be consequently affected by aberrant actin configuration in a cell, as was visible in the Arp2/3 mutants. However, we don’t know if the abnormal morphology and behaviour of ER in mutants like pah1/pah2 would also affect the actin arrangement. Also, myosin localization experiments in the mutants of
Arp2/3 complex could be helpful for studying the role of myosins in ER appearance and dynamics.
5. References


- 99 -


Appendix A - Recipes

Appendix A contains recipes and formulations for all the solutions and media mentioned in the material and method section

Bacterial growth media

**YEB broth-1L, for Agrobacterium**

- 5 g BBL™ Beef extract (Becton, Dickinson & Company, NJ, USA)
- 1 g Yeast extract (Bio Basic Inc. Canada)
- 5 g Peptone-A (Bio Basic Inc. Canada)
- 5g Sucrose (Bio Basic Inc. Canada)
- 0.3g MgSO$_4$.7H$_2$O (Fisher Scientific, Nepean, ON)

Stir to dissolve all the components. Adjust volume to 1 L. Autoclave to sterilize (Autoclaving temperature - 121°C). Store at 4°C.

**YEB solid agar plates- for Agrobacterium**

Before autoclaving the YEB broth, add 15g Difco Bacto-Agar (Becton, Dickinson & Company, NJ, USA). Cool the solution to 50°C before adding antibiotic solution. Pour around 25 ml per 100 X 15 mm petri dishes and allow the plates to solidify before inverting and storing at 4°C.

Antibiotic stock solution

**Kanamycin 50 mg/ml**

- 500 mg kanamycin sulphate salt (Fisher scientific)
- 10 ml ddH$_2$O

**Gentamycin 25 mg/ml**

- 250 mg gentamycin sulphate salt (Fisher scientific)
- 10 ml ddH$_2$O

**Rifampicin, 50 mg/ml**

- 500 mg rifampin (Fisher scientific)
- 10 ml methanol
Dissolve salt into the solution by vortexing. Aliquot into 1.5 ml microfuge tubes and store at -20°C

*Agrobacterium* competent cells

$20\text{mM CaCl}_2$ – 10mL

29.4 mg calcium chloride (Fisher Scientific)

10mL ddH$_2$O

*Plant culture*

1L- 1/2MS media

2.2 g Murashige-Skoog modified basal medium with Gamborg vitamins (Phytotechnology laboratories, USA)

30 g sucrose

850 ml milliQ water

Dissolve components into 850 ml of milliQ water. Adjust pH to 5.7-5.8 using KOH or HCl. Adjust the volume to 1L. Add 3 g/L phytagel (Sigma Aldrich). Autoclave to sterilize and allow to cool down before pouring approx. 25 ml per 100 x 15 mm petri dish. Allow the plates to solidy before inverting and storing at 4°C.

*Arabidopsis* transformation (floral dip method)

Resuspension solution- 500 ml

25 g sucrose

0.5 ml/L Silwet L-77 (Helena chemical company; Collierville TN)

500 ml ddH$_2$O

Dissolve sucrose in ddH$_2$O. Add Silwet-77

MS media with different phosphate concentrations - 3 L

300 mL Murashige and Skoog basal salt micronutrient solution (10x; Sigma Aldrich, Canada)
3 mL Gamborg’s vitamin solution (1000x; Sigma Aldrich, Canada)
30 mL ammonium nitrate, 100x stock (NH₄NO₃) (Sigma Aldrich, Canada)
30 mL calcium chloride dihydrate, 100x stock (CaCl₂·H₂O) (Sigma Aldrich, Canada)
30 mL magnesium sulphate heptahydrate, 100x stock (MgSO₄·7H₂O) (Sigma Aldrich, Canada)
30 mL potassium nitrate, 100x stock (KNO₃) (Sigma Aldrich, Canada)
90 g sucrose

Dissolve all the components together in one solution and adjust the volume to 1500 mL with milliQ water. Divide 1500 mL solution into equal volumes of 500 mL in three individual flasks and name the flasks as 1, 2 and 3. Add 10 mL of potassium phosphate, 100x stock (KH₂PO₄) to the flask 1, 20 ml to the flask 2 and 0 mL to the flask 3. Adjust the pH for each to 5.7-5.8 using KOH or HCL. Bring the volume of each flask to 1L using milliQ water and then add 8 g of agarose (Bioshop, Canada) into each flask. Autoclave to sterilize and allow to cool to 50°C before pouring approx. 25 mL per 100x 15 mm petri dishes. Allow plates to solidify before inverting and storing at 4°C

**Macronutrient stock solutions for the formulation of MS media with different phosphate concentrations- 100x**

165 mg/mL Ammonium nitrate (NH₄NO₃) - 100 mL
16.5 g ammonium nitrate
100 mL milliQ H₂O

44 mg/mL Calcium chloride (CaCl₂·2H₂O) - 100 mL
4.4 g calcium chloride
100 mL milliQ H₂O

37 mg/mL Magnesium sulphate (MgSO₄·7H₂O) - 100 mL
3.7 g magnesium sulphate
100 mL milliQ H₂O

17 mg/mL Potassium phosphate (KH₂PO₄) - 100 mL
1.7 g potassium phosphate
100 mL milliQ H₂O

190 mg/mL Potassium nitrate (KNO₃)- 100 mL

19 g potassium nitrate

100 mL milliQ H₂O

Dissolve salt in milliQ water. Filter sterilize the salt solution with 0.22 uM filters (Fisher Scientific). Store at room temperature.
## Appendix B - Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD1</td>
<td>Actin binding domain 1</td>
</tr>
<tr>
<td>ABD2</td>
<td>Actin binding domain 2</td>
</tr>
<tr>
<td>ABI</td>
<td>Abl-interactor</td>
</tr>
<tr>
<td>ABP</td>
<td>Actin binding protein</td>
</tr>
<tr>
<td>ADF</td>
<td>Actin depolymerizing factor</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine di-phosphate</td>
</tr>
<tr>
<td>APRC5</td>
<td>Actin related protein component 5</td>
</tr>
<tr>
<td>ARP 2/3</td>
<td>Actin related protein 2/3</td>
</tr>
<tr>
<td>ARPC1</td>
<td>Actin related protein component 1</td>
</tr>
<tr>
<td>ARPC2</td>
<td>Actin related protein component 2</td>
</tr>
<tr>
<td>ARPC3</td>
<td>Actin related protein component 3</td>
</tr>
<tr>
<td>ARPC4</td>
<td>Actin related protein component 4</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>CAP</td>
<td>Cyclase associated protein</td>
</tr>
<tr>
<td>Crk</td>
<td>Crooked</td>
</tr>
<tr>
<td>CX</td>
<td>Calnexin</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>Dis1</td>
<td>Distorted 1</td>
</tr>
<tr>
<td>Dis2</td>
<td>Distorted 2</td>
</tr>
<tr>
<td>Dis3</td>
<td>Distorted 3</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl methanesulfonate</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Ermo1</td>
<td>Endoplasmic reticulum morphology 1</td>
</tr>
<tr>
<td>Ermo2</td>
<td>Endoplasmic reticulum morphology 2</td>
</tr>
<tr>
<td>Ermo3</td>
<td>Endoplasmic reticulum morphology 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FABD</td>
<td>F-actin binding domain</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FH1</td>
<td>Formin homology domain1</td>
</tr>
<tr>
<td>FH2</td>
<td>Formin homology domain2</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Gold36</td>
<td>golgi defects 36</td>
</tr>
<tr>
<td>Grl</td>
<td>gnarled</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HSPC300</td>
<td>Haematopoietic stem-cell progenitor</td>
</tr>
<tr>
<td>Hsr3</td>
<td>high sugar response3</td>
</tr>
<tr>
<td>Kam1</td>
<td>katamari1</td>
</tr>
<tr>
<td>Kam2</td>
<td>katamari2</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Klk</td>
<td>klunker</td>
</tr>
<tr>
<td>Lat-B</td>
<td>Latrunculin-B</td>
</tr>
<tr>
<td>Ler</td>
<td>Landsberg erecta</td>
</tr>
<tr>
<td>mEosFP</td>
<td>monomeric Eos fluorescent protein</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige-Skoog medium</td>
</tr>
<tr>
<td>msec</td>
<td>milli sec</td>
</tr>
<tr>
<td>mTalin</td>
<td>mouse Talin</td>
</tr>
<tr>
<td>mvp1</td>
<td>modified vacuole phenotype3</td>
</tr>
<tr>
<td>NAP1</td>
<td>Nck associated protein 1</td>
</tr>
<tr>
<td>Pah1/2</td>
<td>phosphatidic acid phosphohydrolase1/2</td>
</tr>
<tr>
<td>PIR121</td>
<td>p53-121F-induced</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>Rhd3</td>
<td>root hair defective3</td>
</tr>
</tbody>
</table>
RTN  Reticulons
RTNL  Reticulon like protein
SCAR  suppressor of cAR
SRA1  Specifically RAC1 associated
VCA  V erproline homology C onnecting A cidic domain
WASP  Wiskott-Aldrich syndrome protein
WAVE  WASP family Verprolin-homologous protein
YEB  yeast extract broth