Control of cell growth in root hairs

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Priya Vijayakumar

2012

A dedication to my loved ones…….

Control of cell growth in root hairs

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Abstract of Thesis submitted for the degree of M.Sc. by Research

During development plant cells acquire different shapes and often these shapes are correlated with their function. To understand how these cell shapes and forms develop, it is necessary to understand the underlying regulatory mechanisms of cell growth. The growth of root hair cells is a good example of cells undergoing localized cell expansion to acquire their final form. In *Arabidopsis* two bHLH transcription factors RHD6 and its target RSL4 have been identified as key regulators of the initiation and elongation phases of root hair (H) cell development. However in order to obtain a better understanding of this RHD6-RSL4 transcriptional pathway controlling root hair growth, the characterization of the immediate targets of RSL4 is necessary. Here I describe the use of a glucocorticoid inducible expression system coupled with the microarray analysis to identify the direct targets of RSL4. Thirty four putative direct target genes of RSL4 were identified. I assessed the requirement of nine of these genes by characterising the phenotypes of plants homozygous for the loss of function alleles. Plants homozygous for loss of function mutations in *AT1G22620/SAC1, AT1G35670/CPK11/CDPK2, AT1G30870/PRX7* and *AT5G03540/EXO70A1* (44% of the genes analysed) exhibited defects in root hair growth. Taken together my results not only identified potential RSL4 target genes but also demonstrated that RSL4 controls root hair growth by regulating genes that encode proteins involved in cell signalling, vesicle trafficking and cell wall modification.

Contents

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Abbreviations

ABRC- *Arabidopsis* Biological Resource Center

ACT-Actin

AXR-AUXIN RESISTANT

bHLH- Basic helix-loop-helix

cDNA- Complementary DNA

CDS-Coding sequence

ChIP-Chromatin immunoprecipitation

Col-0- Columbia-0

CPC- CAPRICE

CPK-Calcium-dependent protein kinase

Cyc- Cycloheximide

Dex- Dexamethasone

DNA- Deoxyribonucleic acid

EGL- ENHANCER OF GLABRA

ETC- ENHANCER OF TRY AND CPC

EXO-Exocyst

EXPA- EXPANSIN

GFP- Green fluorescent protein

GL- GLABRA

GR- Glucocorticoid receptor

H cell- Hair-bearing cell

KJK- KOJAK

LB- Luria-Bertani

LP- Low phosphate

LRX- Leucine rich repeat extensin

m RNA- messenger RNA

mins- minutes

Mock treatment-Ethanol

MS- Murashige and Skoog

MT- Microtubule

N cell- Non hair-bearing cell

NADPH- Nicotinamide adenine dinucleotide phosphate

NASC- Nottingham *Arabidopsis* Stock Centre

PCR- Polymerase chain reaction

PI-Phosphoinositide

PtdIns(3)P- Phosphatidylinositol 3-phosphate

PtdIns(3,5) P_2- Phosphatidylinositol-3,5-bisphosphate

PR-Pathogenesis related

PRP-Proline- rich protein

PRX-Peroxidase

qRT-PCR- Real-time quantitative RT-PCR

RHD- Root hair defective

RHS-Root hair specific

RIC-Rop interactive CRIB motif containing protein

RNA- Ribonucleic acid

ROP- Plant specific Rho GTPase

ROS- Reactive oxygen species

RSL- RHD-SIX-LIKE

RT-Room temperature

RT-PCR- Reverse transcription PCR

SAC-Suppressor of actin

SCM- SCRAMBLED

SCN-SUPERCENTIPEDE

T-DNA- Transfer DNA

TRH-TINY ROOT HAIR

TRY- TRIPTYCHON

TTG- TRANSPARENT TESTA GLABRA

UTR- Untranslated region

WER-WEREWOLF

XET-Xyloglucan endotransglycosylase

Chapter 1
INTRODUCTION

1. Introduction

Plant cell shape is an important determinant of cell function. Although most cells are isodiametric when they first form in meristems, they acquire different shapes over the course of development. How do these plant cells grow into different shapes during development? The regulation of the cell growth process is the focus of the research in this thesis.

During plant development cells acquire their final form as a result of differential growth (Mathur, 2004). While animal and yeast cells double in their size during development, plant cells typically exhibit one hundred to thousand times increase in their original size (Sugimoto-Shirasu and Roberts, 2003). This increase in plant cell size occurs in two distinct phases: mitotic growth and post mitotic growth. During mitosis the proliferating cells enlarge as a result of an increase in the total cytoplasmic macromolecular mass. Post mitotically, in the absence of cell division, cells then expand by a process that involves an increase in cell volume through vacuolation and a concomitant increase in cell surface (Sugimoto-Shirasu and Roberts, 2003). Post mitotic growth may be classified based on the site at which growth takes place; into diffuse growth and tip growth. Diffusely growing cells such as the trichomes and pavement cells exhibit dispersed growth over a large area of the cell surface whereas during tip growth the cell expansion is localized to a narrow region at the tip of the cells (Mathur and Hulskamp, 2001). In angiosperms root hairs and pollen tubes undergo tip growth.

1.1 Post mitotic cell expansion is an integration of several coordinated cellular events

During post mitotic growth, plant cells coordinate cell expansion through polarized growth. An increase in vacuolar volume caused by the osmotic uptake of water creates a rise in internal turgor pressure that in turn drives global cell expansion by maintaining the stretching of the cell wall (Mathur and Hulskamp, 2001; Guimil and Dunand, 2007). As turgor pressure builds up inside a cell, its counterforce the tensile stress within the cell wall also rises. This stress causes the bonding between the cell wall polymers to loosen enabling them to slip by each other and thus increase the wall surface area. Although turgor is uniform throughout the cell, wall stress is not and depends on the thickness and other mechanical properties of the cell wall. Thinner patches of the cell wall yield to wall stress quicker than thicker areas leading to localized growth. Once an area of the cell surface has been selected for expansion it is followed by wall loosening events catalysed by cell wall modifying enzymes and changes in the cytoskeleton allowing regional cell expansion (Figure 1.1).

An intricate cytoskeletal (microfilaments and microtubules) interaction occurs during differential growth that coordinates the focus of the cell growth mechanism at the cell surface. Microtubules (polymers of tubulin) control the directionality of growth by orientating the fine microfilaments (polymers of actin) in tip growing cells such as root hairs (Whittington et al., 2001; Thitamadee et al., 2002). Actin microfilaments align in the sub apical region of the growing tip and play an essential role in the delivery of the Golgi-derived secretory vesicles to the apical plasma membrane (Miller et al., 1999). The secretory vesicles contain new cell wall precursor materials that are

delivered to the growing plant cell wall. Furthermore the exocytosis of these secretory vesicles is facilitated by the maintenance of a tip focused Ca^{2+} gradient which in turn is regulated by the production of reactive oxygen species (ROS) (Cardenas, 2009). On the whole these findings indicate that several processes such as the modification of the cell wall, actin-based membrane trafficking and the movement of ions function together in a co-ordinated manner during the growth of the expanding cell.

Studying post mitotic growth mechanism in a single cell type, for example root hair (H) cells as in this thesis, will provide an in depth understanding of the mechanisms that control cell growth. Root hairs serve as a good model system to study post mitotic cell growth because of their accessibility, well defined architecture and the ease with which mutants with defective root hair development can be screened and characterised (Carol and Dolan, 2002). A mechanistic understanding of the regulatory pathways controlling root hair growth could serve as a framework to understand cell growth in general. Furthermore given that root hairs undergo tip growth, any insight into the growth mechanisms in this cell type will be instructive in understanding the development of other cell types that undergo tip growth in diverse groups of eukaryotes such as pollen tubes of plants, fungal hyphae and rhizoids in brown algae (Campàs and Mahadevan, 2009).

The main focus of this chapter is to review what is known about the important regulatory mechanisms underlying tip growth, with emphasis on root hair cell development. In the first section, I will outline the different stages and the

mechanisms underlying root hair cell development. In the second part, I will elaborate on a transcriptional regulatory cascade that has been identified to regulate root hair growth in a temporal way.

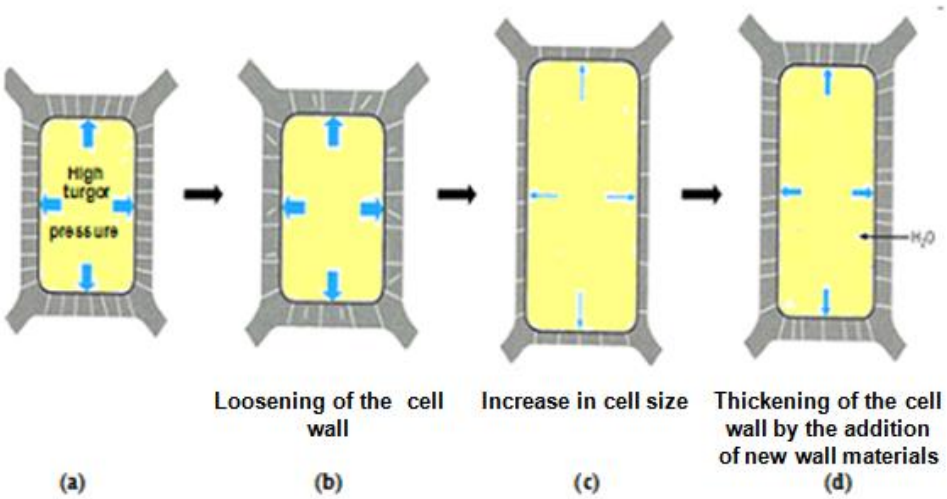


Figure 1.1: Turgor pressure, cell wall loosening and osmotic uptake of water mediate cell expansion.

a) High turgor pressure drives cell expansion b) As the cell begins to expand, the loosening of the cell wall catalyzed by wall loosening enzymes occurs c) The stretching of the longitudinal cell wall causes the thinning of the wall and simultaneous decrease in turgor pressure d) Decrease in turgor pressure triggers the osmotic uptake of water into the cell along with the remodeling of the cell wall. New wall material is added in order to regain the thickness of the wall. Blue arrows indicate turgor pressure. Adapted from (Smith et al., 2009).

1.2 *Arabidopsis* root hairs are a good model to study post mitotic cell growth

Root hairs are tubular outgrowths that differentiate from the apical end (root ward) of the root epidermal hair cells (also referred to as H cells). These specialized hair cells function to increase the surface area of the root in order to facilitate the absorption of water and nutrients, provide anchorage and to interact with soil microorganisms (Guimil and Dunand, 2007). Root hairs undergo cell expansion by means of tip growth. The simple cellular organization and small size makes the root hairs an ideal system to understand plant cell growth.

The root hairs of *Arabidopsis thaliana* have been used as a model to study H cell development because they provide various advantages for developmental, physiological and molecular genetic studies (Grierson and Schiefelbein, 2002). The external localization of the hairs on the root permits easy visualization and analysis of all types of mutants defective in root hair development (Dolan et al., 1994; Galway et al., 1994). Furthermore, root hairs appear rapidly after seed germination, and are not required for the viability and fertility of the plant thereby enabling experimental manipulation. In *Arabidopsis* the development of root hairs occur in a progressive manner in the epidermal hair cells organized in files arising from the root tip (Figure 1.2). This provides the opportunity to analyse all the stages of hair cell development at any given time along the root (Dolan et al., 1994; Grierson and Schiefelbein, 2002). Together these characteristics make *Arabidopsis* root hairs serve as useful tools to study plant cell growth.

Root hair development in *Arabidopsis* is divided into two main stages: fate determination of epidermal cells either as hair (H) cells or non-hair (N) cells and root hair morphogenesis. In *Arabidopsis*, the root epidermis comprises of alternate files of H and N cells whose cell fate is determined at an early stage during development and the two epidermal cell types can be distinguished from each other on the basis of their size.

Upon exiting the division zone, the H cells elongate in the vertical direction (in the direction of the long axis of the root). Once the cells have stopped growing in this direction they produce a small bulge at their root ward end (Carol and Dolan, 2002). Subsequently this bulge extends by tip growth into a root hair perpendicular to the H cell in the maturation zone (Figure 1.2). At this stage, the tip growing hair cells have a characteristic polarized cytoplasmic organization with the accumulation of golgi-derived vesicles at the bulge apex. These vesicles contain cell wall and membrane components that fuse with the plasma membrane. Behind the tip, the sub apical region comprises of the zone rich in organelles such as the endoplasmic reticulum, golgi apparatus and mitochondria (Foreman and Dolan, 2001; Carol and Dolan, 2002). These organelles synthesize and transport macromolecules that maintain the polarized tip growth. The vacuole occupies the basal region of the growing hair cell. In addition, the nucleus moves into the growing bulge of the root hair cell and lodges itself adjacent to the side wall at the beginning of root hair elongation (Ketelaar et al., 2002). On the whole during development the root hair cell traverses through several stages beginning with the specification of the cell fate in the meristem, to the initiation and elongation of the root hair and finally to the cessation of growth .

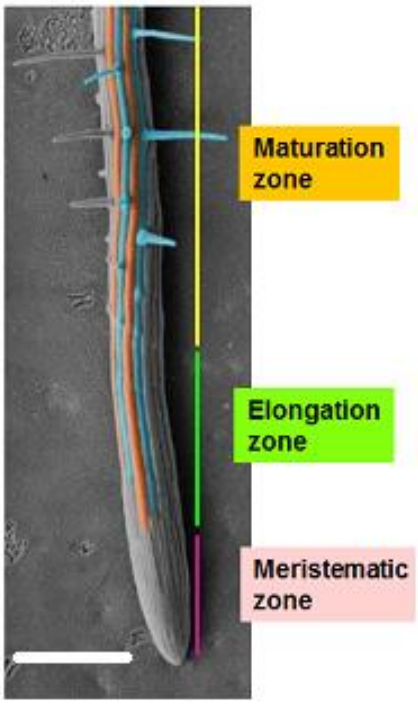


Figure 1.2: Scanning electron microscope (SEM) image of the *Arabidopsis* primary root showing the different zones of epidermal cell development.

Red lines indicate the non-hair (N) cell files that alternate with the hair cell files (H) shown in blue. Scale bar , 500µm. Modified from (Yi, 2008)

1.3 Positional cues define cell fate in *Arabidopsis* root epidermal cells

In mature *Arabidopsis* roots the epidermal cell fate is determined based on their position relative to the underlying cortex. Epidermal cells that overlie two cortical cells develop into hair cells (H), while the cells that overlie a single cortical cell preferentially differentiate into non-hair cells (N) (Figure 1.3) (Dolan et al., 1993; Dolan et al., 1994). Marked differences between the future H cells and N cells even before the emergence of root hairs suggest that the epidermal cell fate is defined early during embryogenesis. Unlike the N cells, the H cells are characterised by a smaller size, greater rate of cell division, reduced vacuolation, enhanced cytoplasmic density and a distinct chromatin organization (Dolan et al., 1993). Positional information as opposed to cell lineage controls the specification of the epidermal cell fate. When either the N cell or H cell is ablated, the neighbouring cell divides and occupies the position of the ablated cell, adopting the identity corresponding to the new position (Berger et al., 1998). This analysis suggested that the position of the epidermal cells relative to the underlying cortex is required for the specification of cell pattern in the root epidermis.

1.3.1 Patterning genes regulate the root epidermal cell identity

In *Arabidopsis*, the root hairs develop from the root epidermis in a predictable fashion. This patterning is generated by a combination of positional signalling and lateral inhibition mechanisms (Parker et al., 2000). Furthermore, several molecular genetic approaches have facilitated the identification of genes that regulate these mechanisms during the H cell fate determination (Figure 1.4) (Schiefelbein et al., 2009).

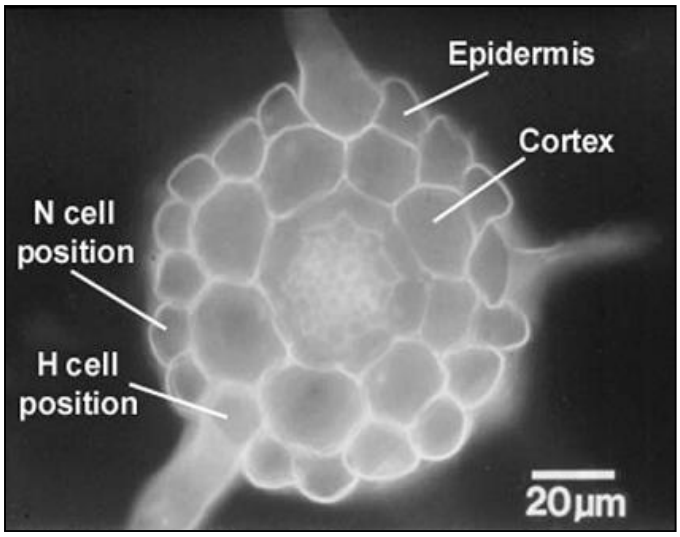


Figure 1.3: Transverse section of the *Arabidopsis* root showing the position dependent patterning of the epidermal hair cells (H) and non-hair cells (N).

Scale bar , 20µm (Grierson and Schiefelbein, 2002).

The identification of SCRAMBLED *(*SCM), a putative trans membrane receptor kinase, helped bridge the gap between the detection of positional cues and the establishment of appropriate cell pattern by root epidermal cells (Kwak et al., 2005). SCM functions to relay positional signals to the epidermal cells by negatively regulating the *WEREWOLF* (*WER*) transcription in the H cells, enabling them to adopt the hair cell fate. WER is a MYB transcription factor and a positive genetic regulator of N cell identity (Lee and Schiefelbein, 1999). Additionally, a WER related MYB transcription factor, MYB23 has been identified to play an essential role in the specification of the non-hair cell identity (Kang et al., 2009). Root epidermal cell fate is governed by a regulatory network that comprises both positive and negative regulators of H cell fate. WER, MYB23, TRANSPARENT TESTA GLABRA 1 (TTG 1, a WD40-repeat protein) (Galway et al., 1994), GLABRA3 and ENHANCER OF GLABRA3 (GL3 and EGL3, related bHLH transcription factors) accumulate in the N cells and promote the non-hair cell fate by activating *GLABRA2 (GL2),* which encodes a homeodomain-leucine-zipper (HD-Zip) transcription factor protein (Schiefelbein, 2000; Schiefelbein et al., 2009).

The TTG1–GL3/EGL3–WER/MYB23 core transcriptional complex positively regulates the transcription of *CAPRICE (CPC)* that encodes a MYB-repeat transcription factor and the related proteins TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC 1 (ETC1) in the N cells. CPC, TRY, and ETC1 then mediate lateral inhibition by moving to the adjacent H cells. In the H cells, CPC competitively binds to the TTG–GL3/EGL3 and promotes the H cell fate (Schiefelbein et al., 2009). CPC binding decreases the GL2 expression and induces the expression of GL3-EGL3

which then translocates to the N cell forming a second lateral feedback loop (Bernhardt et al., 2005). Once the patterning mechanism specifies the identity of the H cells in the root epidermis they undergo differentiation to give rise to mature root hairs.

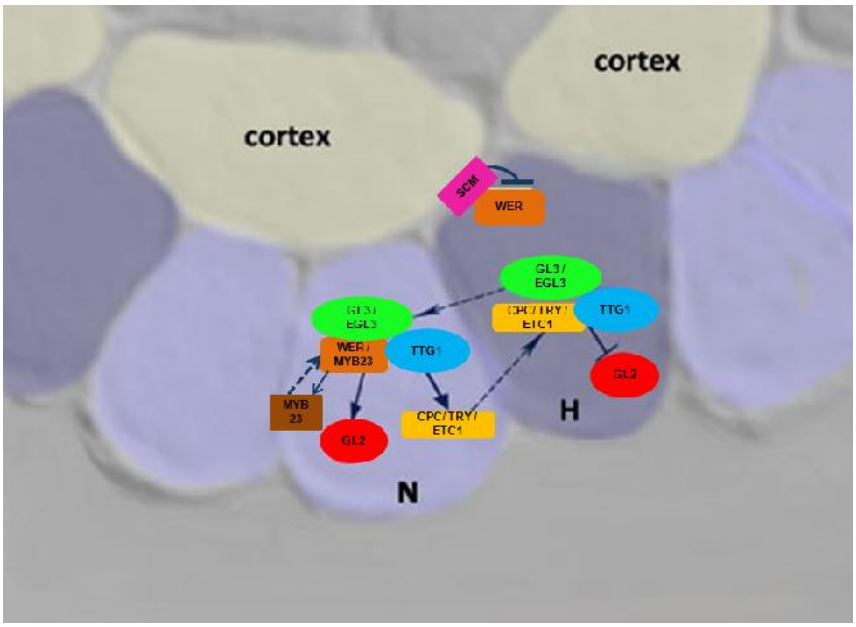


Figure 1.4: Model showing the gene network regulating the root epidermal cell specification in *Arabidopsis.*

(N) Non-hair cells and (H) hair cell. Arrows indicate positive control, and blunted lines indicate negative regulation; solid lines show gene/protein regulation and dashed lines represent the movement of proteins. Figure modified from (Datta et al., 2011).

1.4 Hair initiation and tip growth occur during root hair cell differentiation

Once the hair cell fate has been determined the root hair develops from the H cell. For simplicity in explaining the physiological and regulatory processes underlying H cell development, the morphogenesis of root hairs can be broadly subdivided into two stages: a) root hair initiation and b) tip growth.

1.4.1 Root hairs initiate from the apical end of the H cell

Root hair initiation begins with the selection of a hair outgrowth site at the root ward end (apical) of the H cell (Figure 1.5b). In *Arabidopsis* genetic screens of mutants that produced root hairs either from a different site or from multiple sites helped identify genes involved in the spatial regulation of the root hair initiation.

Fewer root hairs develop in *root hair defective 6 (rhd6)* mutants compared to that of the wild type plants and those that do form show a shoot ward (basal) shift in the emergence of the root hairs. Because *rhd6* mutations are recessive, these phenotypes suggest that *RHD6* is required for root hair initiation and for the specification of the position of hair initiation to a single site in the H cell (Masucci and Schiefelbein, 1994). Additionally transgenic lines that over express ROP GTPases like ROP2 develop defective hairs (Jones et al., 2002). These proteins localize to the future site of hair outgrowth indicating that they could be involved in root hair initiation (Figure 1.5 b) (Molendijk et al., 2001). ROP GTPases (Rho-related protein from plants) function as molecular switches that direct cell growth via a myriad of regulatory and signal transduction pathways. ROP2 is regulated by RhoGTPase GDP dissociation inhibitor (RhoGDI) encoded by *SUPERCENTIPEDE1 (SCN1*). Phenotypic characterization of

scn1 mutants showed that in addition to ROP2 being mislocalized, they developed multiple hair initiation sites (Carol et al., 2005). Furthermore on over expressing ROP2 the plants produced multiple misplaced hairs, adding further evidence to the role of ROPs in site selection. Together, these data indicate that RhoGDI regulated ROPs have a role in restricting the hair initiation to a single site.

Hormones like auxin and ethylene play an important role in root hair initiation. According to Masucci and Schiefelbein (1996), auxin and ethylene regulate root hair development downstream of the patterning genes (such as *WER, CPC,*) (Masucci and Schiefelbein, 1996). The *AUXIN RESISTANT2 (AXR2)* gene was identified as an important component of the auxin response pathway that affects the root hair initiation. *axr2,* a gain-of-function mutant exhibited a decrease in the number of root hairs along with a basal shift in root hair position (Wilson et al., 1990). Additionally a similar phenotype was observed in mutant plants defective in auxin transport (*aux1* and *trh1)* indicating that the auxin influx contributes towards the root hair positioning (Fischer et al., 2007). Along with auxin, ethylene also modulates the polar positioning of root hairs. *etol* mutant, which overproduces ethylene showed a more apical shift in hair emergence when compared to wild type plants (Guzman and Ecker, 1990). In contrast there is a more basal shift in the site of root hair outgrowth in the *ethylene-resistant* (*etr1)* mutant which is relatively insensitive to ethylene (Masucci and Schiefelbein, 1994), emphasizing on the importance of ethylene in the proper positioning of the hair emergence site.

Once the site for the root hair emergence has been determined, a small region of the cell wall loosens leading to the formation of a bulge. One of the major physiological changes occurring during bulge initiation is the acidification of the cell wall along with

the simultaneous elevation in cytoplasmic pH at the initiation site (Bibikova et al., 1998). This acidification of the cell wall is predicted to enhance cell wall loosening as some wall enzymes are activated with a decrease in pH (McQueen-Mason et al., 1992). Expansins are a group of non-hydrolytic cell wall loosening proteins that are activated at low pH (Cosgrove, 2000). In *Arabidopsis*, *EXPANSIN7 (EXPA7)* and *EXPANSIN 18 (EXPA18*) were identified, that are specifically expressed in the H cells suggestive of their role in root hair differentiation through wall loosening (Cho and Cosgrove, 2002). Further Lin et al (2011) demonstrated that a decrease in *EXPA7* transcript reduced root hair growth providing in vivo evidence for the function of EXPA7 in root hair development (Lin et al., 2011).

Together these results indicate that root hair cell initiation is characterized by the coordination of several physiological processes.

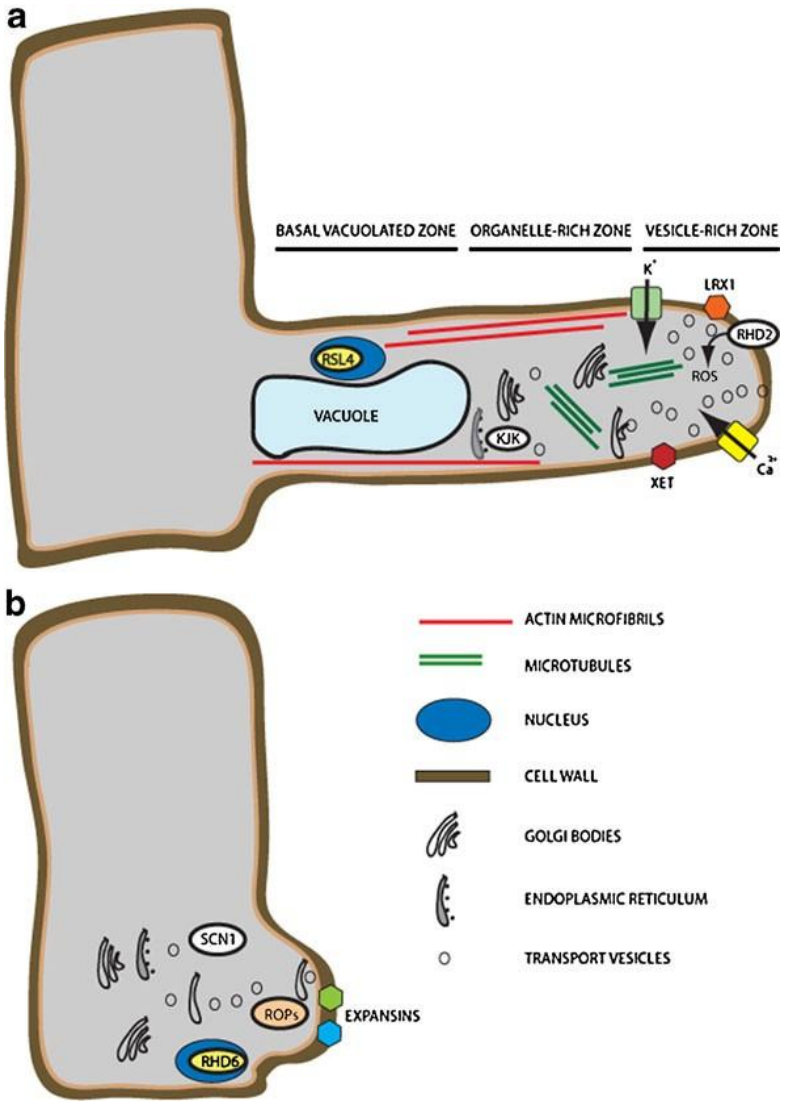


Figure 1.5: Schematic representation of some of the physiological and regulatory processes involved in the root hair (H) cell (a) tip growth and (b) initiation.

(Datta et al., 2011)

1.4.2. Root hairs undergo unidirectional tip growth

Following initiation, the root hairs elongate unidirectionally by tip growth and acquire their mature form (Figure 1.5a) (Dolan et al., 1994). During tip growth, along with the organization of a polarized cytoplasm, the nucleus is maintained at a fixed distance from the apex. Ketelaar et al (2002) showed that this nuclear positioning in growing H cells is dependent on actin filaments. Furthermore this actin controlled nuclear localization was shown to be essential for root hair growth, as the basipetal migration of the nucleus resulted in growth arrest (Ketelaar et al., 2002). Together these studies show that the actin dependent nuclear positioning in H cells is an absolute requirement for the maintenance of H cell elongation.

As well as forming a physical link between the nucleus and the H cell apex, the actin filaments also facilitate the transport of secretory vesicles, enclosing newly synthesized wall materials to the growing tip. Further evidence implying the role of these microfilaments in tip growth was based on pharmacological studies done with latrunculin B (an inhibitor of actin polymerization). Treatment with this actin depolymerising drug was shown to arrest tip growth of *Arabidopsis* root hairs (Baluska et al., 2000). Likewise a similar phenotype was observed in the loss of function mutants of the *ACTIN 2 (ACT 2*) gene, encoding an actin family protein. *act-2* mutants developed short and swollen root hairs (Nishimura et al., 2003). Taken together these results confirm the indispensable role of actin during root hair elongation.

Microtubules (MT) are also involved in root hair growth. Treatment of root hairs with taxol (a MT stabilizing drug) or oryzalin (a MT depolymerising drug) results in the reorientation of hair growth, leading to the development of wavy and branched hairs

(Bibikova et al., 1999). In addition to the loss in directionality of the growing tips, tip focussed Ca^{2+} gradient is directed to each of these branched growing points in taxol-treated root hairs. This data suggests that MT's spatially control the tip focussed Ca^{2+} accumulation in root hairs and thereby the direction of hair growth itself.

Bibikova et al (1997) showed that during root hair growth a high concentration of Ca^{2+} is maintained at the tip region when compared to the rest of the cell (Bibikova et al., 1997). ROOT HAIR DEFECTIVE 2 (RHD2), a NADPH oxidase regulates tip growth by ROS (reactive oxygen species) production which in turn induces a tip focussed Ca^{2+} influx by activating the hyperpolarisation activated Ca^{2+} channels (Foreman et al., 2003; Ishida et al., 2008; Takeda et al., 2008**).** This elevated cytoplasmic Ca^{2+} in turn activates the RHD2 NADPH oxidase by binding to its EF hand forming a positive feedback loop (Takeda et al., 2008). Furthermore, RHD2 is also required for the maintenance of the cell wall integrity. *rhd2* mutants develop bulges that do not elongate into root hairs but instead some of the mutant hairs burst due to the unstable cell wall (Macpherson et al., 2008). Together these data indicate that interplay between RHD2, ROS and Ca^{2+} underlies root hair cell growth.

During root hair growth the translocation of potassium, an osmotically active ion is essential. Observation of the root hair phenotypes of K+ transporter and channel mutants (*akt1 and trh1-1*) revealed their distinct roles in the uptake of potassium during tip growth (Rigas et al., 2001; Desbrosses et al., 2003). AKT1 (*Arabidopsis* K + transporter 1*)*, an inward rectifying potassium channel was identified as being responsible for potassium transport during hair elongation (Desbrosses et al., 2003). Subsequently TRH1 (TINY ROOT HAIR1*)*, a potassium transporter was implicated in potassium translocation. *trh1* mutants show an arrest in growth after initiation indicating

that TRH1 is a positive regulator of root hair elongation (Kim et al., 1998; Desbrosses et al., 2003). Thus, the uptake of potassium by K+ transporters like AKT1 and TRH1 is vital for root hair elongation.

Root hair growth is thought to be accompanied by the remodelling of the cell wall. The plant cell wall comprises of a complex network of polysaccharides (cellulose microfibrils embedded in a matrix of pectin and hemicellulose) and structural proteins that undergo enzyme catalyzed rearrangement during growth (Levy and Staehelin, 1992). Xyloglucan endotransglycosylases (XET) are cell wall modifying enzymes that modify xyloglucan (hemicellulosic wall polysaccharide) chains during tip growth (Nishitani and Tominaga, 1992). Additionally along with the modification of the existing cell wall components, new wall materials are synthesized and delivered to the growing cell wall. *KOJAK (KJK)* encoding a cellulose synthase like (subfamily D) protein has been suggested to function in the synthesis of non-cellulosic cell wall polysaccharides. *kjk* mutants fail to produce root hairs as the H cells rupture soon after initiation due to the defect in cell wall modification (Favery et al., 2001). In addition to polysaccharides, structural proteins eg: extensins, have been shown to control tip growth of root hairs. *LRX1* encodes a leucine rich extensin protein that is localized to the cell wall of H cells. *lrx1* knockdown mutants develop hairs that are either branched or swollen or fail to elongate. This implies that LRX1 regulates tip growth via cell wall synthesis (Baumberger et al., 2001). Together all these data highlight the importance of cell wall modification during root hair tip growth.

A number of bHLH transcription factors regulating hair cell development have been discovered. RHD-SIX-LIKE-4 (RSL4) was identified as a direct target of RHD6

and was found to be a key regulator of hair growth. *rsl4* loss of function mutants exhibited shorter and lesser hairs while on the other hand constitutive expression of RSL4 resulted in the development of very long root hairs (Yi et al., 2010). Hence these observations indicate that RSL4 is a key regulator of root hair growth. Further characterization of the targets of RSL4 might help gain insight into the regulatory mechanisms underlying tip growth in root hair cells.

1.5 Transcriptional regulation of the root hair cell development

During plant development the control of the cell fate specification and growth, play a crucial role in determining the morphogenetic processes that give rise to the final shape and size of individual cells. In *Arabidopsis*, the developmental stages that a single H cell undergoes to give rise to the mature root hair have been well documented (Dolan et al., 1994). However, not much was known about the transcriptional regulatory network underlying the root hair (H) cell development. The RSL family of bHLH transcription factors was implicated to function at different stages of H cell development (Yi, 2008). The attribution of gene regulators to the multiple phases of H cell development has been possible by the analysis of mutant lines of each of these RSL family genes.

1.5.1 A highly conserved RSL family of transcription factors control root hair development

ROOT HAIR DEFECTIVE 6 (RHD6) encoding a basic helix-loop-helix (bHLH) transcription factor was identified as a regulator of H cell development (Masucci and Schiefelbein, 1994; Menand et al., 2007). Basic helix–loop-helix (bHLH) proteins belong to a family of transcriptional regulators that have been well characterized across eukaryotic organisms. These transcription factors are defined by the bHLH domain which consists of around 60 amino acids and comprises of two distinct segments: the basic region and the helix loop helix region (HLH). While the basic region is involved in DNA binding, the HLH region is essential for the dimerization between bHLH proteins (Pires and Dolan, 2010). These proteins have been found to be involved in a number of developmental processes such as cell proliferation and the differentiation of specific cell types (Massari and Murre, 2000; Toledo-Ortiz et al., 2003; Pires and

Dolan, 2010). In *Arabidopsis* a closely related bHLH family of genes have been shown to be required for the specification and differentiation of stomatal cells. MacAlister et al (2007) showed that in *Arabidopsis*, SPEECHLESS (SPCH), a stomatal bHLH transcription factor and two of its paralogues are necessary for the establishment of the stomatal cell lineage (MacAlister et al., 2007).

Similarly *RHD6* along with other *RSL* genes (the *RSL family* genes) have been implicated in root hair cell development (Yi et al., 2010). The *RSL* genes form a transcriptional regulatory network that temporally controls the root hair initiation and elongation by tip growth.

1.5.2 RHD6 is a positive regulator of the early stages of root hair cell differentiation

In *Arabidopsis RHD6* belongs to the subfamily VIIIc of bHLH transcription factors (Heim et al., 2003). In addition to *RHD6* this subfamily comprises of five other members: *RSL (RHD SIX LIKE) 1, 2, 3, 4,* and *5* that are collectively known as the *RSL* family genes. This subfamily is further classified into VIIIc1 (Class I) and VIIIc2 (Class II) (Figure 1.6) (Pires and Dolan, 2010).

Massuci and Schiefelbein (1994) were the first to identify that ROOT HAIR DEFECTIVE 6 (RHD6) is involved in the establishment of root epidermal cell polarity via site selection at the apical end of the H cell of the root epidermis. Their conclusion was based on observing the root hair phenotype of the *rhd6* loss of function mutants in *Arabidopsis*. *rhd6* mutants exhibit a basal shift in the emergence of root hairs along with fewer and shorter root hairs when compared to that in the wild type (Figure 1.7B) (Masucci and Schiefelbein, 1994). The gene was subsequently cloned and the defect in

root hair development was identified as due to the mutation in the *RHD6* gene, *rhd6* mutants were complemented with a whole gene *RHD6p::GFP:RHD6* translational fusion with green fluorescent protein (GFP) (Menand et al., 2007). As a result of this complementation RHD6 protein was found to accumulate in the H cell nuclei in the meristem and elongation zones of the *rhd6* mutant roots but disappeared before the emergence of the root hair (Figure 1.7C). These results suggest that RHD6 controls the H cell differentiation at an early stage by regulating other genes that encode proteins that are functional during the actual process of hair initiation and growth.

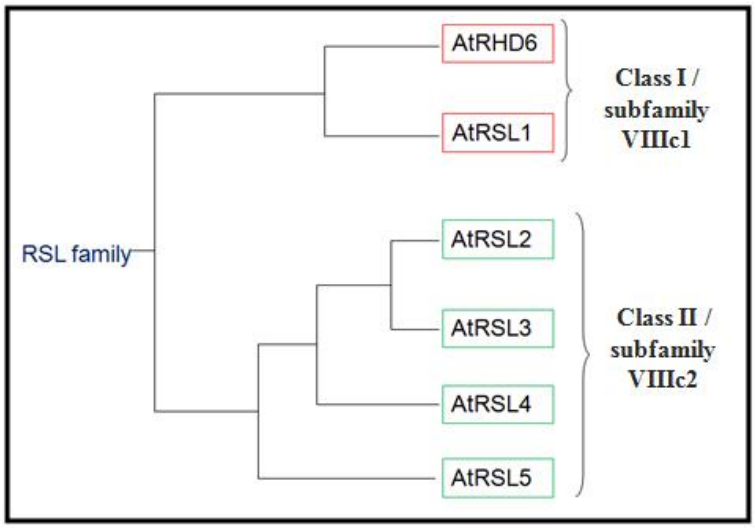


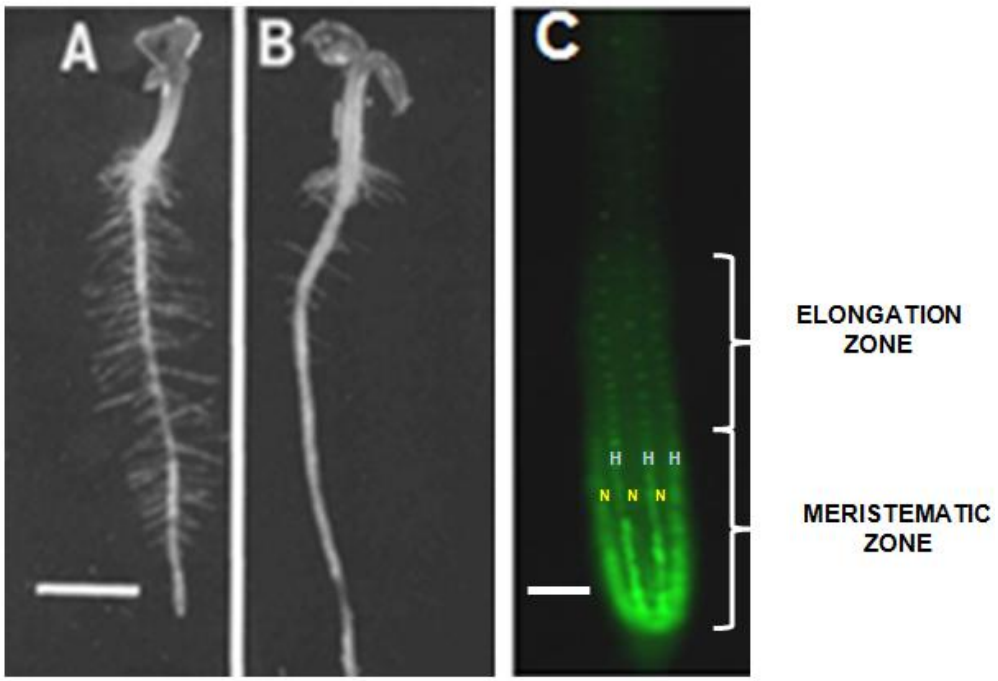
Figure 1.6: Schematic representation of the *Arabidopsis* RSL transcription factor family.

RHD6 along with RHD-SIX-LIKE (RSL1) belong to the RSL Class I / subfamily VIIIc1 and RSL2, RSL3, RSL4 and RSL 5 belong to the Class II / subfamily VIIIc2.

1.5.3 RHD6 and RSL1 function redundantly during root hair development

RSL1 is the most closely related gene to *RHD6* suggesting that this gene could also be functional during root hair development (Menand et al., 2007; Pires and Dolan, 2010). Although the single loss of function mutant of *RSL1* exhibited the wild type root hair phenotype, the *rhd6 rsl1* double mutant showed a severe root hair phenotype as it did not produce any root hairs (Menand et al., 2007). These observations indicate that both *RHD6* and *RSL1* redundantly regulate root hair morphogenesis.

Furthermore expression studies done using *rhd6 rsl1* mutant plants harbouring the *GFP:RSL1* fusion construct showed that RSL1 accumulated in the nuclei of the H cells in the meristem and elongation zone of the roots similar to RHD6 (Figure 1:7). Additionally in order to determine if RHD6 and RSL1 are specifically needed for root hair development, Menand et al (2007) observed the phenotypes of other tip growing cells: pollen tubes in the *rhd6, rsl1,* and *rhd6 rsl1* mutants. As no defect was observed they concluded that RHD6 and RSL1 function redundantly only during root hair development (Menand et al., 2007). Together these results suggest that RHD6 and RSL1 function together to positively regulate root hair cell development.



Col-0 ***rhd6*** ***RHD6p: GFP: RHD6***

Figure 1.7: *rhd6* loss of function mutants develop short root hairs; RHD6 is expressed in the hair cells of the meristem and the elongation zone of the root.

A: Wild type seedling exhibiting normal root hair morphology (4 day old Col-0). **B:** *rhd6* mutant seedling showing a decrease in the number of root hairs. **C:** Expression of the genomic GFP fusions of RHD6 in root hair cells. Scale bar, A and B: 1mm, C: 200µm (Masucci and Schiefelbein, 1994; Yi, 2008).

1.5.4 RSL4 is a key regulator of root hair tip growth

Following the establishment of H cell identity and the initiation, the root hair elongates by tip growth. This post mitotic growth process defines the final size and shape of the H cell. However little was known about the transcriptional regulators of tip growth in H cells until the characterization of a bHLH transcription factor, ROOT HAIR DEFECTIVE 6-LIKE 4 **(**RSL4).

Yi et al (2010) discovered RSL4 as an immediate target of the previously defined bHLH transcription factor RHD6 (ROOT HAIR DEFECTIVE 6) by using a glucocorticoid (GR) inducible expression system (Yi et al., 2010). *rhd6 rsl1* mutant plants were transformed with the steroid hormone [dexamethasone, (Dex)] inducible, translational fusion of RHD6 to the rat glucocorticoid receptor (GR) hormone binding domain (GR:RHD6). *RSL4* was not found to be expressed in the *rhd6 rsl1* mutants transformed with GR:RHD6 unless treated with Dex. After 2 hours of Dex treatment, the *RSL4* gene expression was induced indicating that RHD6 activates the transcription of its downstream target *RSL4* and root hair growth initiates (Yi et al., 2010). However in order to confirm that RSL4 is a direct target of RHD6 the effect of Cycloheximide (Cyc), on the expression of *RSL4* was tested. Cyc is a protein synthesis inhibitor that blocks the synthesis of indirect target proteins thereby facilitating the identification of the immediate target genes (Sablowski and Meyerowitz, 1998). The induction of the *RSL4* transcription was found to be insensitive to Cyc treatment indicating that RSL4 is an immediate downstream target of RHD6 (Yi et al., 2010). To determine the role of RSL4 in regulating hair growth, the root hair phenotype of the loss of function mutant (*rsl4*) was characterized. The mutants exhibited a defect in root hair development: fewer

and shorter root hairs when compared to the wild-type plants (Figure 1.9) (Yi et al., 2010) implicating RSL4 to be a key regulator of root hair growth.

RHD6 being a positive regulator of RSL4 was found to accumulate in the nuclei of H cells in the meristem and elongation zones, suggesting that RSL4 would also accumulate in these H cells. As expected RSL4 was present in the H cells in the elongation zone prior to the emergence of the root hair and is also found to accumulate in the hair cells throughout the growth phase (Figure 1.8C) (Yi et al., 2010). Together these results indicate that RSL4 is expressed in tip growing H cells downstream of the RHD6, implying that RHD6 and RSL4 temporally regulate root hair development

To test if *RSL4* would be sufficient to promote root hair growth, RSL4 was constitutively expressed *(CaMV 35S:RSL4*) in wild type (Col-0) plants. The root hairs of the over expression plants (*CaMV 35S:RSL4)* exhibited continuous growth until they reached the stage where wild type hairs died. Thus the hairs of the plants transformed with *CaMV 35S:RSL4* were approximately 4-5 times longer than that of the wild type before the occurrence of cell death (Figure 1.8B). Based on these observations Yi et al (2010) concluded that the constitutive expression of RSL4 resulted in an increase in the growth phase of H cells till cell death (Yi et al., 2010). This suggests that RSL4 is a key regulator of root hair growth.

Another bHLH transcription factor, RSL2 which is 40% identical to the RSL4 protein was predicted to positively regulate root hair growth. In addition to being expressed simultaneously with *RSL4, RSL2* is also regulated by RHD6 and RSL1 (Yi et al., 2010). Furthermore the phenotypic analysis of the *rsl2* mutants which showed shorter hairs than wild type plants was indicative of the role of RSL2 in root hair

development (Figure 1.9). While the single *rsl2* and *rsl4* mutants developed shorter hairs, the *rsl2 rsl4* double mutant exhibited a severe root hair defect as they failed to produce any hairs (Figure 1.9). This result showed that the absence of *RSL2* enhanced the *rsl4* mutant phenotype further supporting the prediction that *RSL2* positively regulates root hair growth. However in contrast to *RSL4* the constitutive expression of *RSL2* did not result in the continuous growth of root hairs leading to the formation of very long hairs suggesting that *RSL2* functions in a less important role than *RSL4* during hair growth. Taken together these data indicate that *RSL2* is also positive regulator of root hair growth.

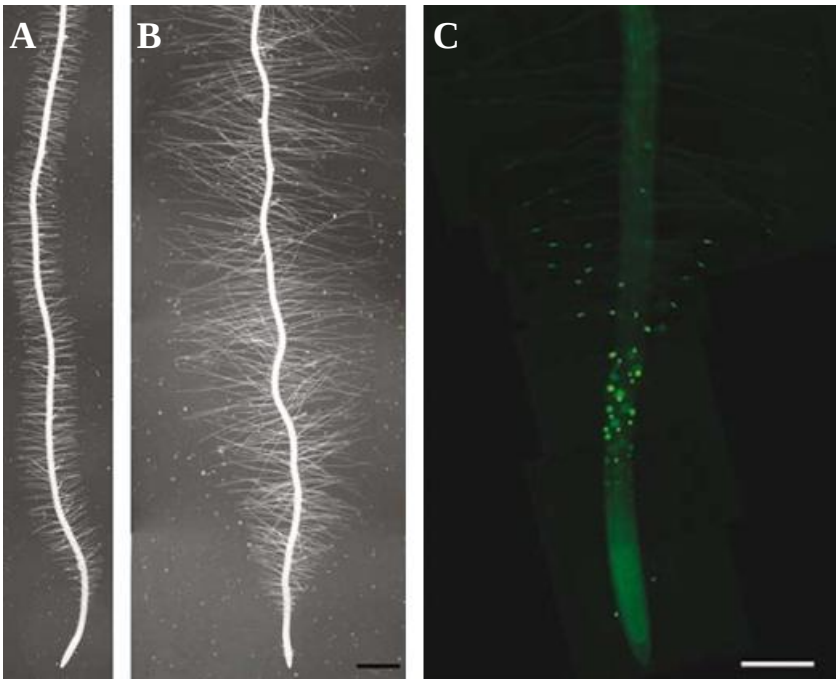


Figure 1.8: Constitutive expression of RSL4 results in the development of long root hairs; RSL4 is expressed in the growing root hairs cells.

A: Wild type seedling exhibiting normal root hair length (4 day old Col-0) **B:** *35S:RSL4* seedling exhibiting an increase in root hair length, **C:** Expression of the genomic GFP fusions of RSL4 in root hair cells. Scale bar, A and B: 1mm, C: 200 µm (Yi et al., 2010).

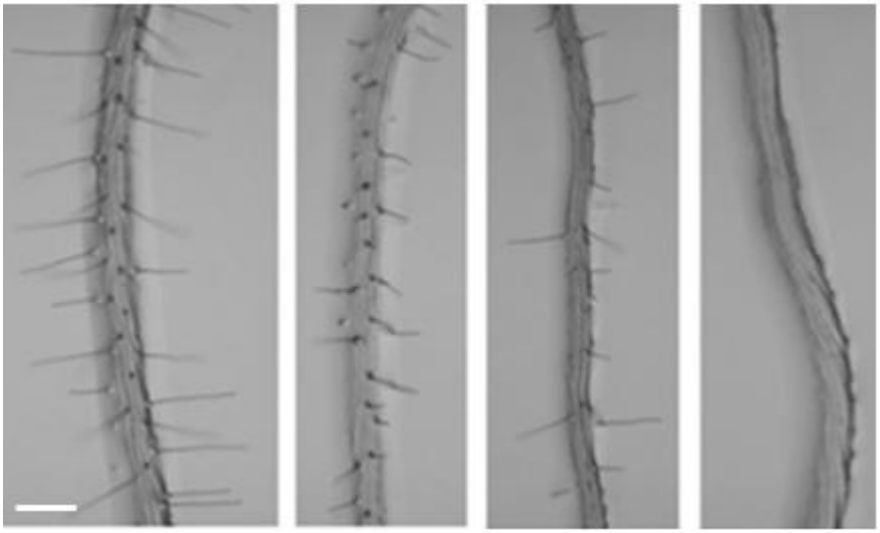


Figure 1.9: Root hair morphology of Col-0, *rsl2*, *rsl4* and *rsl2 rsl4* mutants.

rsl2, *rsl4* single mutants develop shorter root hairs. *rsl2 rsl4* double mutant exhibits severe defect in root hair development and is hairless. Scale bars, 200 µm (Yi, 2008).

1.5.5 RSL4 regulates root hair growth by integrating endogenous developmental and exogenous environmental signals

Auxin is a hormonal effector that promotes hair elongation in *Arabidopsis* by acting downstream of the *RSL* Class I genes (*RHD6, RSL1*). Masucci and Schiefelbein (1994) observed that hormones like auxin did not influence the H or N cell fate determination in the *Arabidopsis* root epidermis (Masucci and Schiefelbein, 1994). On the other hand both exogenous and endogenous auxin sources affect the H cell morphogenesis (Pitts et al., 1998). Exogenous auxin treatment of *rhd6* and *rhd6 rsl1* mutants restores root hair growth suggesting that auxin activates growth by acting downstream of RHD6 and RSL1. In order to verify this hypothesis the levels of *RSL4, (*a downstream target of RHD6) upon auxin treatment were examined both in the wild type (Col-0) and in the *rhd6 rsl1* mutant plants (Yi et al., 2010). As expected auxin increased the *RSL4* gene expression in the *rhd6 rsl1* double mutants indicating that auxin treatment is sufficient to stimulate the expression of *RSL4* even in the absence of *RHD6* and *RSL1* gene function. Further evidence was obtained on treating *rsl2 rsl4* double mutant plants with auxin. Auxin did not restore hair elongation in these mutants indicating that auxin acts via RSL4 to enable root hair growth. Hence taken together, these data demonstrate that *RSL4* activity is required for auxin stimulated root hair growth.

Similarly, low phosphate (LP) stress enhances the root hair elongation by modulating the expression of *RSL4.* Availability of LP significantly increases root hair length along with a rise in *RSL4* expression (Yi et al., 2010). Together, these data indicate that RSL4 is the central player that integrates both the internal and external signals for root hair tip growth (Figure 1.10).

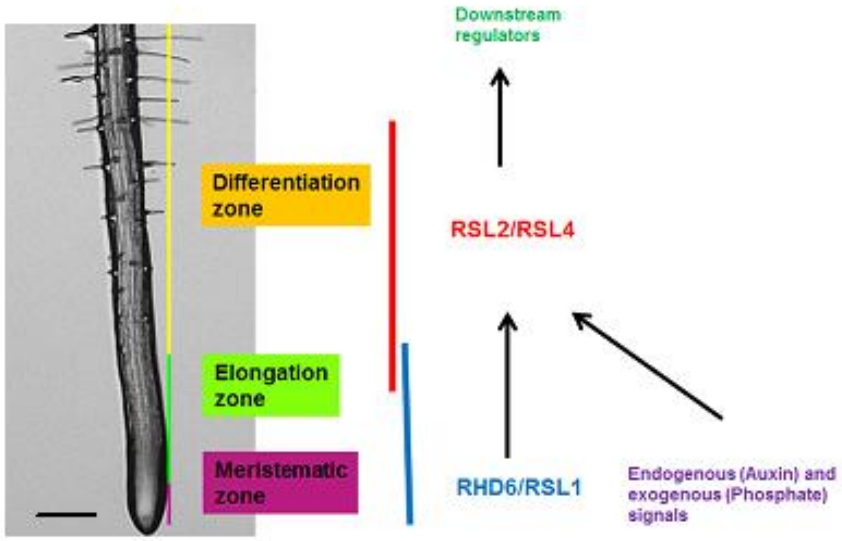


Figure 1.10: RSL4 functions as a master regulator of cell growth by integrating endogenous developmental and exogenous environmental signals.

RHD6 / RSL1 accumulate in the meristematic and elongation zone as indicated by a blue line; RSL4 / RSL2 are present in the elongation zone just before hair outgrowth and also in the growing root hair till growth ceases as shown by a red line. Scale bar, 500 µm.

Further RSL4 has been predicted to regulate H cell elongation through the transcriptional control of genes that encode proteins affecting cell growth. In order to test this hypothesis the transcriptomes of wild-type, *rsl4* mutant and *CaMV 35S:RSL4* roots were compared. Based on this microarray analysis, Yi et al (2010) identified 83 genes with reduced expression in *rsl4* mutants and elevated expression in *CaMV 35S:RSL4* plants. A few examples are *EXPANSIN 7(EXPA7), EXPANSIN 18 (EXPA 18)* and *PROLINE RICH PROTEIN 3 (PRP3*) (Yi et al., 2010).

Out of the putative target genes nearly 20% have been found to be root hair specific and also implicated in root hair growth, thereby indicating that *RSL4* positively regulates growth by activating the expression of genes (such as those encoding cell wall modifying proteins) that affect cell extension (Yi et al., 2010). Further characterisation of these genes will be required to determine if RSL4 controls development by regulating the expression of genes required for cell growth.

Aim and Objective

A combination of physiological, genetic and molecular approaches have helped gain a better understanding of the distinct processes occurring at each stage of hair cell (H) development. Although in the recent past some of the key regulators of hair cell initiation (RHD6) and elongation (RSL4) have been identified, a complete transcriptional regulatory cascade is yet to be defined. Understanding the core machinery that controls H cell development will serve as a blue print that could be used to study similar growth mechanisms across diverse organisms and also facilitate genetic manipulations for agricultural benefits

The present study was used to obtain a clear picture of the genes directly regulated by RSL4 in order to promote root hair cell growth. I adopted two approaches to explore the mode of action of *RSL4* transcription and identify its downstream target genes.

As part of the first study using a glucocorticoid inducible expression system, I identified several potential RSL4 direct target genes that encode proteins that function in various physiological processes like cell wall modification, vesicle trafficking and signal transduction. These data show that RSL4 regulates root hair development by regulating various cell growth processes.

Additionally as part of an independent study I selected a subset of potential RSL4 target genes from the microarray data done by comparing the transcriptomes of wild type, *rsl4* and *35S:RSL4* roots for further functional characterisation (Yi et al., 2010). The loss of function mutants and overexpression lines of the selected genes did not exhibit any alteration in root hair development indicating these genes are not crucial regulators of H cell development.

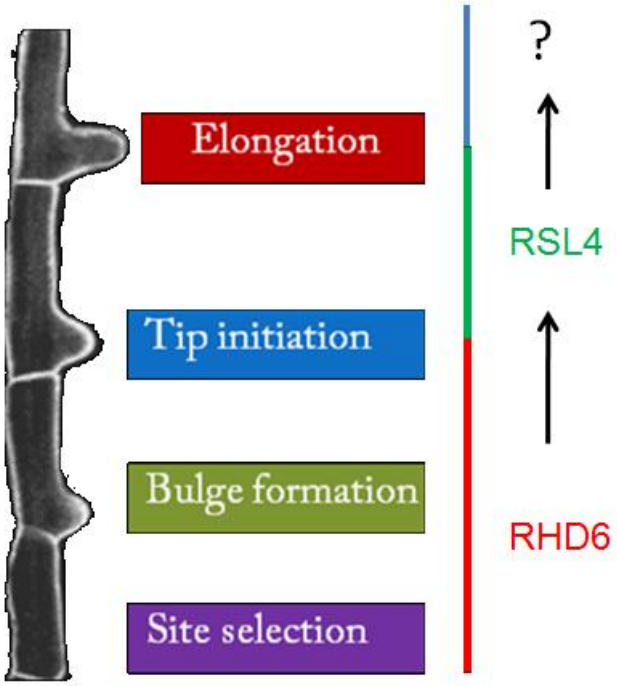


Figure 1.11: Aim of the project: To define the RHD6-RSL4 transcriptional regulatory pathway underlying root hair development by identifying the immediate target genes of RSL4.

Chapter 2:
MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

2.1.1 Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana)* ecotype Columbia (Col-0) was used for all the experiments. The T-DNA insertion mutant seeds were obtained from the *Arabidopsis* Biological Resource Centre (ABRC) and the Nottingham *Arabidopsis* Stock Centre (NASC). The *Arabidopsis* seeds were surface sterilized in 70% ethanol for 5 minutes followed by 5% (v/v) bleach for 10 minutes and rinsed with sterile distilled water thrice.

For the T-DNA insertion lines and the glucocorticoid (GR) inducible lines the seeds were plated on Paul's medium with 0.7% Phytagel (w/v) (Sigma,UK) (Recipe in Appendix 7). In order to observe the root hair phenotype of the overexpression lines the plants were grown vertically at a 45 ° angle in half-strength Johnson's medium with 0.5% Phytagel (w/v) (Sigma,UK) (Recipe in Appendix 8).

After a period of stratification at 4°C for 2 days the plants were grown vertically under continuous illumination at 25°C in the growth chamber. The roots were observed 7 days after sowing.

2.1.2 Bacteria and growth conditions

For the recombinant DNA work the *Escherichia coli* strain DH5α was used and *Agrobacterium tumefaciens* was used for the *Arabidopsis* transformation.

E.coli was cultured at 37°C in Luria Bertani medium (LB) containing 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride, either in liquid or on 1% agar plate with the suitable antibiotics.

A.tumefaciens was grown on YEB medium at 28°C with gentamycin along with the appropriate antibiotic for selecting the transformants**.** YEB medium contained 0.5% (w/v) beef extract, 0.5% (w/v) peptone, 0.5% (w/v) sucrose, 0.1 % (w/v) Yeast Extract and 0.5% (w/v) Magnesium Sulphate Heptahydrate.

2.1.3 Chemicals

The chemicals used were supplied by Sigma Aldrich (Dorset, UK), Fischer scientific (USA), Melford (Suffolk,UK) and VWR (Belgium)

2.1.4 Restriction Enzymes

Restriction enzymes were obtained from Fermentas (York,UK) and from New England Biolabs (Hitchin,UK).

2.1.5 Oligonucleotide primers

The PCR reaction primers were acquired from Sigma-Genosys (Cambridge,UK) . Sequences can be found in the Appendix.

2.1.6 Antibiotics

All antibiotics were prepared as a 1000x stock solution and filter sterilized and stored at -20°C. The appropriate concentrations they were used in are as shown in the table below.

Table 1: Concentration of antibiotics used.

Antibiotic	**Stock**	**Final Concentration**		
		E.coli	***A.tumefaciens***	**Plants**
Rifampicin	50 mg/ml MeOH	150 µg/ml	50 µg/ml	_____
Hygromycin	50 mg/ml	_____	25 µg/ml	50 µg/ml
Gentamycin	50 mg/ml H_2O	15 µg/ml	25 µg/ml	_____
Kanamycin	100 mg/ml H_2O	10-50 µg/ml	50 µg/ml	50 µg/ml

2.2 Methods

Cloning

2.2.1 Generation of constructs

2.2.1.1 GR fusion vector

In order to generate the N-terminal GR fusion vector, the multisite gateway system was used. The gateway entry clone vectors: pDONR P4-P1R containing the endogenous promoter plus the 5'UTR of the *RSL4* gene, pDONR P2R-P3 cloned with the coding region of the *RSL4* gene plus 3'UTR and terminator and the GR domain inserted into the p DONR 207 vector, were obtained from the lab (Yi et al., 2010). Then a Gateway LR multisite reaction (Gateway LR clonase reaction mix, Invitrogen) was carried out with pDONR P4-P1R, pDONR P2R-P3, pDONR 207-GR plus the binary destination vector pGWB multisite in order to generate the GR:RSL4 fusion vector. Primers used to check the GR:RSL4 constructs are listed in Appendix 1. The binary vector pGWB multisite was a gift from Matt Tomlinson (John Innes Centre, Norwich) and was generated by replacing the R1-CmR-ccdB-R2 cassette of pGWB1 into R4-CmR-ccdB-R3 (pGWB1 is from Tsuyoshi Nakagawa, Shimane University, Japan). This construct was then transformed into *rsl4* and *rsl2 rsl4* mutant backgrounds (Construct in Appendix 2). After screening for the primary transformants the selected plants were grown for another generation. The T2 lines were then plated onto selective media, out of the seeds plated 75% survived (25% homozygous, 50% heterozygous). These seedlings were then transferred to the soil. The homozygous T3 lines were selected based on the 100% survival of the seedlings in the antibiotic supplemented plates. These lines were used for further experiments.

2.2.1.2 Overexpression vectors

For the overexpression constructs, the coding sequences (CDS) of the candidate genes (*AT4G04900, AT4G13390, AT5G57530, AT1G34510, AT4G30320* and *AT1G26250*) were PCR amplified from the root cDNA obtained from 15 day old Col-0 seedlings. The primer list for the generation of the over expression constructs are listed in Appendix 6. Subsequently the PCR fragments were cloned either into BamHI and SalI sites or KpnI and SalI sites of a modified pCAMBIA1300 plasmid containing the CaMV 35S promoter and the terminator of pea Rubisco small subunit E9 from 35S: pCAMBIA1301 cloned into its EcoRI and PstI site. The resulting overexpression plasmids were then transferred into the Agrobacterium strain GV3101 which was then used to transform into *Arabidopsis*. The homozygous lines were obtained based on antibiotic screening similar to the GR lines.

2.2.2 Plant transformation

Agrobacterium mediated floral dip method (Clough and Bent, 1998) was used to transform all the binary vectors into *Arabidopsis.* Plants with a few siliques and several immature buds were used for transformation. 250 ml of *Agrobacterium tumefaciens* culture incubated at 28 °C overnight was spun down and the pellet was resuspended in 200ml solution containing 5% (w/v) sucrose and 0.05% (v/v) Silwet L-77. The floral tissues of the *Arabidopsis* plants were dipped into the bacterial mixture for 30 seconds after which the plants were laid down on a tray with wet tissues and covered with cling film overnight. The dipped plants were then returned to the green house.

2.2.3 Primary transformant screening

To select the GR:RSL4 transformed lines, the T1 plants were screened on MS Agar plates supplemented with 50 µg/ml kanamycin and 50 µg/ml hygromycin and the overexpression lines were selected on MS Agar plates containing 50 µg/ml hygromycin.

2.2.4 Plant treatments

2.2.4.1 Dexamethasone treatment

20mM stock solution of Dexamethasone (Dex) in ethanol was used for the treatment. T2 seeds of the GR:RSL4 *rsl2 rsl4* lines were at first germinated on MS agar plates containing 50 µg/ml hygromycin and after 4 days the seedlings were transferred to MS medium without antibiotics. 6 to 12 day old seedlings were flooded with 20ml MS liquid media containing 20µl Dex (Dex treatment), 20ml MS liquid media with 20µl ethanol (Mock treatment). After treatment the root tips were sampled at several time points for the q RT-PCR and microarray analysis

2.2.4.2 Cycloheximide treatment

Cycloheximide (Cyc) was dissolved in ethanol to make the 20mM stock solutions to be used in the experiments. Similar to the Dex treatment 6 to 12 day old seedlings of *rsl2 rsl4* harbouring the inducible RSL4 protein driven from the native promoter and terminator were used for the cycloheximide treatment. Both Dex and Cyc were added from stock solutions in ethanol (20mM). The seedlings were flooded with 20ml MS liquid medium with 20 µl Dex and 20 µl Cyc (Dex + Cyc treatment). After treatment the root tips were sampled at several time points for q RT-PCR and microarray analysis. Additionally for the Cyc treatment the seedlings were treated with 20ml MS liquid media containing 20µl Cyc alone.

2.2.5 Characterization of the root hair phenotype of the T-DNA insertion mutant lines

The T-DNA insertion mutants of the putative target genes obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) and *Arabidopsis* Biological Resource Centre (ABRC) were first genotyped in order to obtain homozygous lines and were then used for further analysis

2.2.5.1 Plant genomic DNA extraction for Genotyping

A small piece of leaf tissue was macerated in liquid nitrogen, followed by which 400µl of extraction buffer (200mM Tris HCl, 250mM NaCl, 25mM EDTA, 0.5% SDS, pH 7.5) was added and vortexed for 15 seconds and placed at room temperature (RT) for 5 minutes. After a brief centrifugation, the supernatant (300 µl) was transferred to a fresh eppendorf tube containing 300µl isopropanol followed by incubation at RT for 2 minutes. Finally after centrifugation at 13,000 rpm for 5 minutes the pellet was dried and the DNA was dissolved in 50µl of Tris EDTA (TE) buffer. 0.5µl of this extracted DNA was used for the genotyping PCR with the reaction volume being 20 µl. Primer pairs used for genotyping are listed in the Appendix 3,4.

2.2.5.2 Mutant and Overexpression line screening

The root hair phenotype of the T-DNA insertion and overexpression lines was characterized by measuring the root hair density and the hair length.

2.2.5.2.1 Root-hair length measurement

To measure the length of root hairs in plants homozygous for the T-DNA insertions, root hairs located around 3mm from the root tip of 7 day old seedlings were imaged under the Leica DFC310 FX camera mounted on a Leica M165 FC stereo microscope. The digital images obtained were used directly for root hair length measurement by the Image J software. Ten seedlings of each genotype were measured.

For the over expression lines, root hairs measuring approximately 10 mm to the root tip were recorded using a Leica DFC310 FX camera mounted on a Leica M165 FC stereo microscope. Twenty five seedlings for each genotype were used for the measurements; approximately 20 of the longest hairs were measured for each seedling.

2.2.2.5.2 Root hair density measurement

Root hairs of 7 day old seedlings were imaged and twenty serial root hair cells per seedling were scored. Ten seedlings of each genotype were measured in the root hair differentiation zone using a Leica M165 FC stereo microscope.

2.2.6 RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA was extracted from root tips (of approximately 1000 seedlings) using the RNeasy Plant Mini Kit (Qiagen, Germany cat#74904) with an on column DNase (Qiagen, Germany cat#79254) treatment step. RNA was then quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized from 450 ng of RNA using the SuperScript III First-Strand Synthesis System for RT-PCR using oligo(dT) (Invitrogen). In order to avoid amplification of genomic DNA, qRT-PCR primers (listed in Appendix 5) were generated in the intron - flanking regions. They were designed to generate PCR products between 100-120bp long. The qRT-PCR analysis was performed with the SYBR Green PCR Master Mix (Applied Biosystems cat#4364344). Each 10 µl qPCR reaction contained 4 µl total cDNA (1:20 dilution), 5 µl SYBR Green Master Mix, and 0.5µl of each primer (10 µM). Reactions were carried out as three replicates per reaction in 96-well Microplates (Applied Biosystems). An initial denaturation step of 10 minutes at 95°C was followed

by 40 cycles of 15 seconds incubation at 95°C, 60 seconds at 60°C and 72°C for 30 seconds. At the end of the reaction, the samples were heated at 72°C for ten minutes. This was followed by the measurement of fluorescence during a dissociation curve in which the temperature was raised from 70°C to 95°C. The specificity of the qPCR reaction was ensured by the detection of gene-specific single peak and by the absence of primer dimer peaks. The ΔΔCt method was used to calculate the relative gene expression. The GAPDH gene (*Arabidopsis*) was used as a reference for normalisation (Vandesompele et al., 2002).

2.2.7 Microarray Hybridization

Gene expression in the GR:RSL4 *rsl2 rsl4* seedling root hairs under different treatments (Mock, Dex, Dex + Cyc) were compared using the Affymetrix Gene Chip *Arabidopsis* ATH1 Genome Array (Santa Clara, CA, USA). Affymetrix microarrays are produced by the synthesis of oligonucleotides 25 bases in length on a quartz surface using photolithography.

Total RNA was isolated from three independent biological replicates. The RNA samples from the root tips of the RSL4 inducible lines under different treatments were then quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA) and assessed using an Agilent 2100 bioanalyzer (Agilent Technologies). cDNA was synthesized from 1μg of total RNA using one-cycle target labelling and control reagents (Affymetrix) to produce biotin-labeled cRNA. Following fragmentation the cRNA was hybridized to the *Arabidopsis* ATH1 Genome Array. Each microarray was washed and stained with streptavidin-phycoerythrin in a Fluidics station 450 (Affymetrix) and scanned at 1.56μm resolution in a GeneChipScanner 3000 7G

System (Affymetrix). All the analysis including the hybridization, staining, washing and screening for quality of the arrays were done at the Genomics Service Center of the Centro Nacional de Biotecnología,Spain (Consejo Superior de Investigaciones Científicas).

2.2.8 Gene expression analysis

For background correction, normalization, and expression level summarization the multi array analysis algorithm was used (Irizarry et al., 2003). Statistical analysis and graphical visualization of data were done using the interactive tool FIESTA http://bioinfogp.cnb.csic.es/tools/FIESTA). In order to determine changes in gene expression an analysis of the variance was used. P values were adjusted by false-discovery rate (FDR) to correct for multiple testing (Benjamini and Hochberg, 1995). Genes with adjusted P values of <=0.001 were selected as statistically significant. They were considered to be differently expressed if the change in expression was >2 fold (up regulated). The values obtained in the Dex treated samples were compared with those obtained from Mock treated samples to identify the genes induced upon RSL4 activation. Further, in order to determine if the gene expression of these RSL4 induced genes are unaffected upon the addition of cycloheximide (Cyc) the values in the Dex + Cyc treated samples were compared with that of the Mock. The genes that exhibited similar values in both the analysis were taken as putative direct target genes of RSL4.

Chapter 3:
RESULTS

3. Steroid inducible form of RSL4 as a tool to study root hair development

3.1Abstract

Root hairs undergo post mitotic cell growth during the development of their final shape and form. Two bHLH transcription factors ROOT HAIR DEFECTIVE 6 (RHD6) and ROOT HAIR DEFECTIVE 6 - LIKE 4 (RSL4) together form part of the transcriptional regulatory network regulating hair cell development. While RHD6 controls the early stages of root hair development, RSL4 has been shown to be a key regulator of hair growth. Characterisation of this RHD6-RSL4 transcriptional network by the identification of genes regulated by RSL4 would facilitate the elucidation of the overall regulatory cascade involved in root hair development. In order to determine the genes regulated by RSL4, I used a glucocorticoid inducible expression system. Here I report the generation of a sensitive steroid inducible version of RSL4 that serves as a molecular switch to identify the downstream targets of RSL4. Upon activation RSL4 induces root hair formation suggesting that RSL4 regulates hair growth right from the hair initiation stage.

3.2 Introduction

In *Arabidopsis* the RSL family of genes encoding the bHLH transcription factors regulate root hair morphogenesis. ROOT HAIR DEFECTIVE 6 (RHD6) was the first member of the RSL family to be identified as a regulator of root hair initiation and was found to function downstream of the patterning genes (Menand et al., 2007). *rhd6*, a T-DNA insertion mutant of *ROOT HAIR DEFECTIVE 6 (RHD6)* exhibits a decrease in the number of root hairs along with a basal shift in the hair emergence site (Masucci and Schiefelbein, 1994). In our lab Yi et al (2010), identified *ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4)* as an immediate target of RHD6. Loss of function mutants (*rsl4*) develop fewer and shorter hairs while the constitutive expression of *RSL4* results in long root hairs implying that RSL4 is a key regulator of cell growth in root hairs (Yi et al., 2010). Preliminary microarray studies done by comparing the transcriptional profiles of wild-type, *rsl4* and *CaMV 35S:RSL4* roots identified a number of genes which were down-regulated in the mutant and up-regulated in the RSL4 overexpressor indicating that they might be functioning downstream of *RSL4*. These included genes encoding structural proteins, Rop GTPase interactors and cell wall modifying enzymes required for root hair growth (Yi et al., 2010). This suggests that RSL4 could promote the hair (H) cell growth by regulating the expression of the downstream genes involved in cell growth processes such as cell wall modification and endomembrane trafficking. Characterisation of these downstream genes may help uncover the complete transcription cascade regulating root hair development. This in turn could serve as a representative of similar regulatory mechanisms that control cell growth across diverse organisms. Since the preliminary transcriptome analysis comparing both the loss and

gain of function mutants of *RSL4* to wild type (Col-0) gave us an extensive list of differentially regulated genes I designed a parallel experiment to validate the previous analysis as well as to identify the immediate targets of RSL4. In plants, the induction of a transcription factor followed by the transcriptional profile analysis has served as a powerful method to identify downstream target genes (Sablowski and Meyerowitz, 1998). Hence the approach I adopted here to identify the RSL4 target genes was based on the detection of the transcript level of genes affected by the induction of RSL4 in the root hairs.

3.3 Results

3.3.1 Use of the inducible form of RSL4 to identify downstream targets

In mammalian cells, the glucocorticoid steroid receptor (GR) functions as a molecular switch that modulates the activity of transcription factors to which they are fused in a glucocorticoid hormone dependent manner (Schena et al., 1991). The GR acts as a signal transducer and interacts with both the hormonal signal (Dexamethasone) and the gene it regulates (Picard et al., 1988). Stancato et al (1996) showed that the fusion of the hormone binding domain of the GR to a transcriptional regulator is sufficient to function as an inducible expression system (Stancato et al., 1996). This steroid receptor based transcription system has been adopted by many investigators to regulate gene expression in plants (Lloyd et al., 1994; Simon et al., 1996).

In *Arabidopsis* the GR inducible version of several transcriptional regulators has enabled the identification of downstream targets that regulate various developmental processes such as the root stem cell organisation and floral organ identity (Sablowski and Meyerowitz, 1998; Levesque et al., 2006). Similarly I utilised the GR inducible expression system to identify the genes regulated by RSL4 during root hair development.

As a first step I generated a steroid regulated version of *RSL4* (*GR:RSL4*) and introduced it into mutants lacking *RSL4* function. After integration into the genome, followed by transcription in the nucleus, the GR:RSL4 fusion protein is synthesized in the cytoplasm. In the cytoplasm the GR:RSL4 protein forms a complex with the heat shock protein 90 (hsp90). Steric hindrance of this complex prevents the nuclear transport of the fusion protein. Dexamethasone (Dex) is a synthetic corticosteroid that mimics the natural steroid hormone and serves as a ligand for the glucocorticoid

receptor (GR). Upon application of Dex, the GR:RSL4 is released from the complex and is transferred into the nucleus (Figure 3.1). This results in the transcription of genes regulated by RSL4. Taken together I devised a system to regulate the nuclear translocation of GR:RSL4 by exogenous chemical treatment thereby controlling the activation of downstream genes.

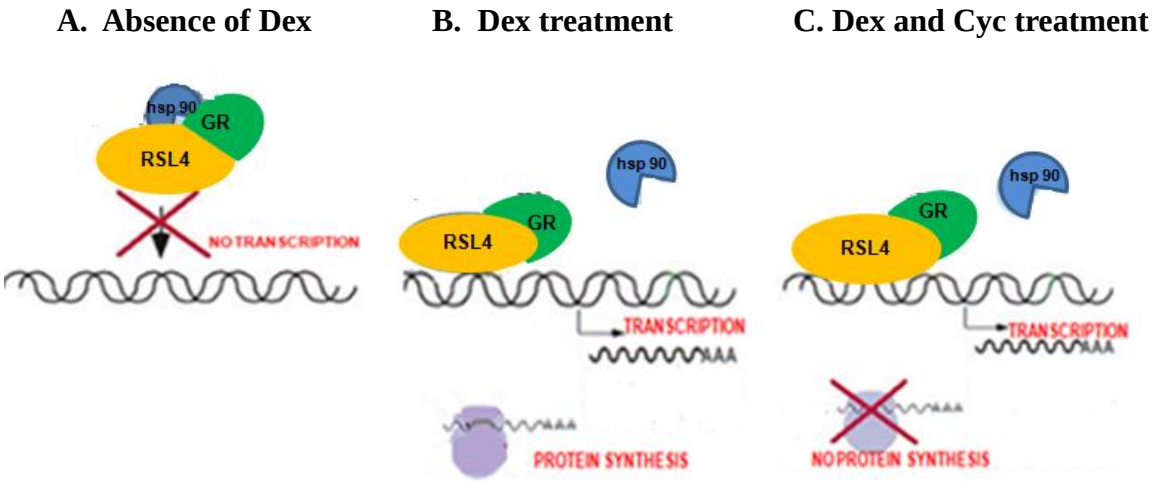


Figure 3.1: Schematic representation of the Dex inducible system that would facilitate the identification of the downstream targets of RSL4.

A: In the absence of dexamethasone (Dex) treatment the GR:RSL4 fusion protein is retained in the cytoplasm as a complex with the heat shock protein 90 (hsp 90). **B:** Upon Dex treatment the GR: RSL4 is released from the complex and translocates into the nucleus bringing about the transcription of its target genes. **C:** Simultaneous treatment with Dex and cycloheximide (Cyc) facilitates the transcription of only the immediate target genes of RSL4 while inhibiting the translation of the protein and subsequent transcription of the indirect target genes of RSL4. Adapted and modified from (Sablowski and Meyerowitz, 1998).

3.3.2 RSL4 induces root hair development upon Dex treatment

To determine if the induction of RSL4 can promote root hair formation I made a genomic glucocorticoid receptor (GR) ligand-binding domain fusion construct for *RSL4* (*GR:RSL4*) that is driven by the native promoter. This construct was then transformed into *rsl4* (data not shown) and *rsl2 rsl4* mutants. However the *rsl2 rsl4* mutants harbouring the GR:RSL4 was used for further experiments as it was easy to observe the root hair development upon RSL4 induction in the otherwise hairless *rsl2 rsl4* mutants. Over one hundred T1 individual lines of *rsl2 rsl4* harbouring GR:RSL4 were obtained. Phenotypic characterisation of T1 seedlings showed a hairless root phenotype similar to the *rsl2 rsl4* mutants under normal growth conditions. Further a subset of these T1 plants (approximately 10 lines) were transferred to Paul's medium supplemented with 20 μM Dex. All the lines showed good root hair complementation upon Dex treatment. These lines were grown for a further two generations in order to obtain homozygous plants. The homozygous T3 seeds obtained were used for further analysis. To confirm the role of RSL4 in root hair growth, I incubated the 12 day old T3 seedlings with MS liquid containing 20 μM Dex. Clear bulges depicting the early stages of root hair formation were seen after 3 - 4 hours of incubation. On further observation at different time points, the initial bulge grew rapidly giving rise to long hairs (Figure 3.2). In addition to developing root hairs in the primary root of the GR:RSL4 *rsl2 rsl4* seedlings (Figure 3.3), long hairs were seen even in the lateral roots highlighting the importance of the RSL4 regulation in the overall plant root hair growth (Figure 3.4).

Further evidence indicating that RSL4 is responsible for root hair development came from observing the root hair phenotype of the GR:RSL4 *rsl2 rsl4* seedlings

incubated in MS liquid without Dex. Even after prolonged incubation (30 hours) in the absence of Dex these seedlings did not develop any root hairs. These data indicate that upon Dex treatment the RSL4 activation promotes root hair growth.

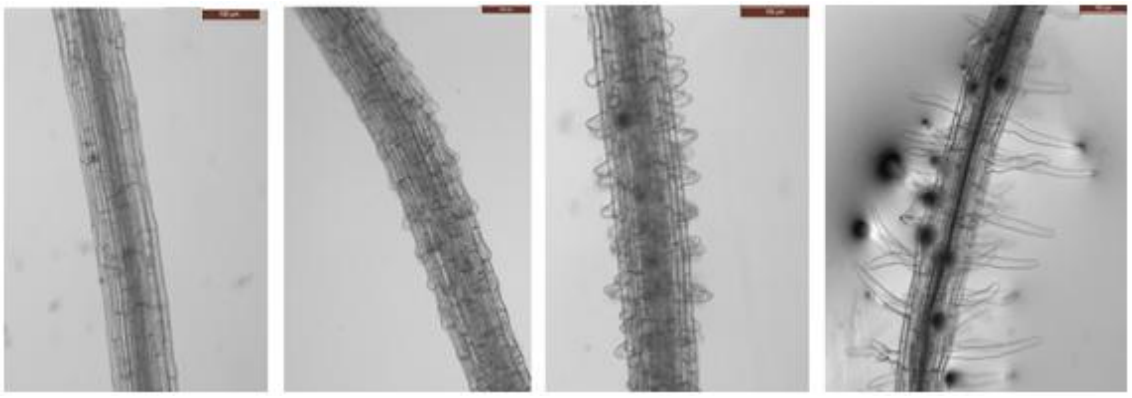


Figure 3.2: Dex induction of GR:RSL4 results in root hair development.

Bright field images taken from the primary root of 12 day old *rsl2 rsl4* seedlings harbouring GR:RSL4 treated with 20 µM Dex at different time points. Scale bars, 100 µm

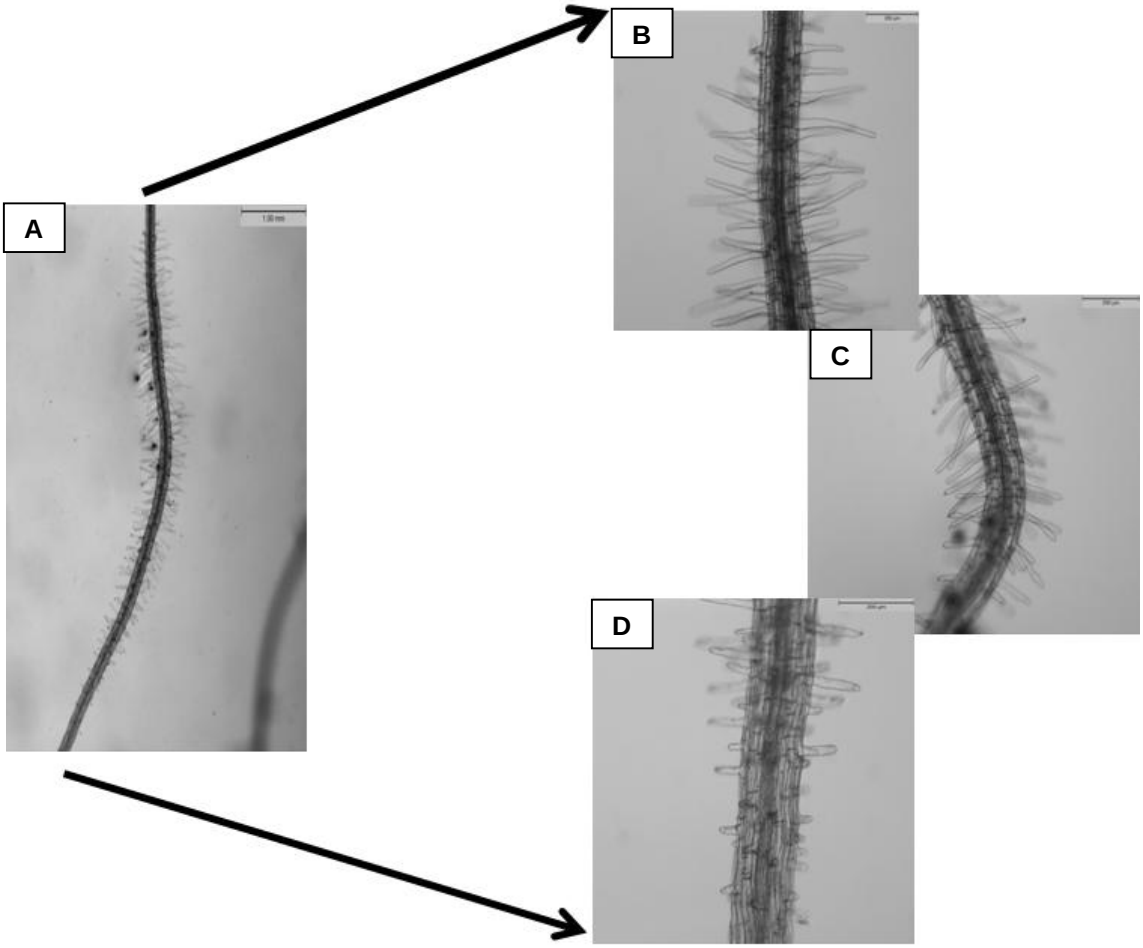


Figure 3.3: RSL4 induces root hair formation in the primary root.

Bright field images of the primary root of a 12 day old *rsl2 rsl4* double mutant seedling harbouring GR:RSL4, 24 hours after Dex treatment. Scale bars, A: 1mm, B, C, D: 200µm

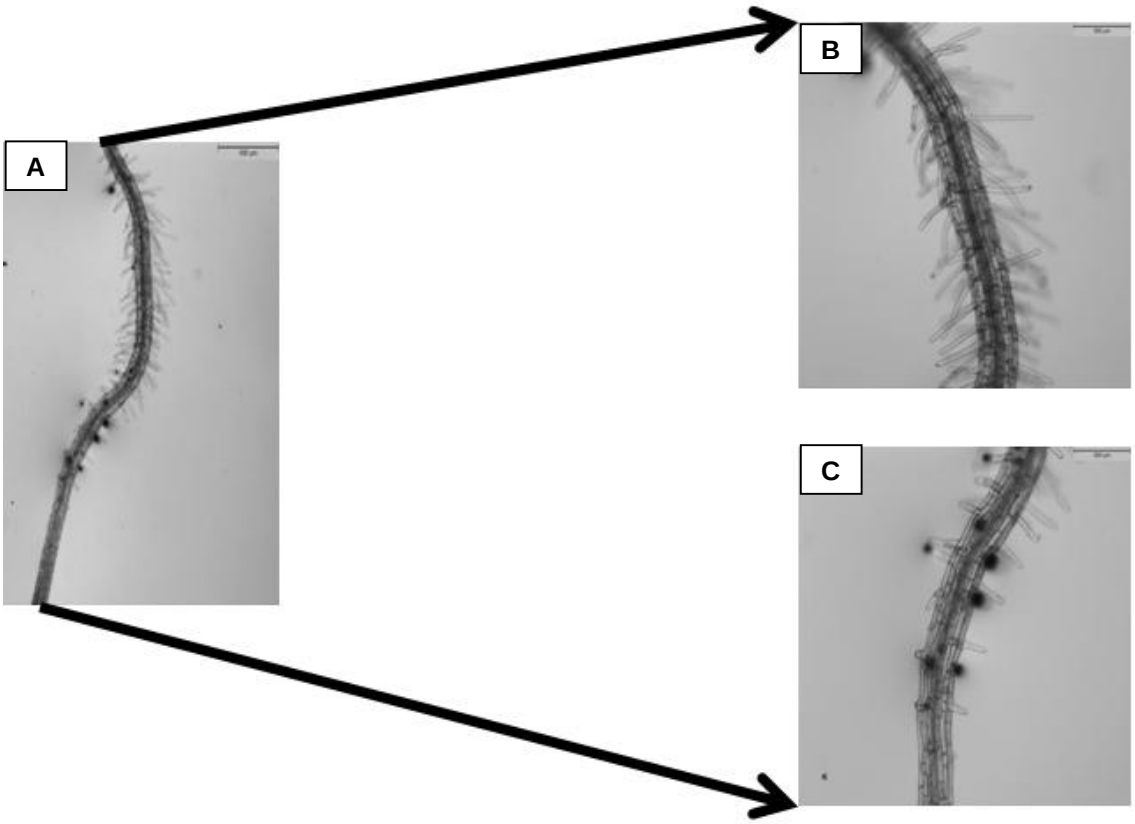
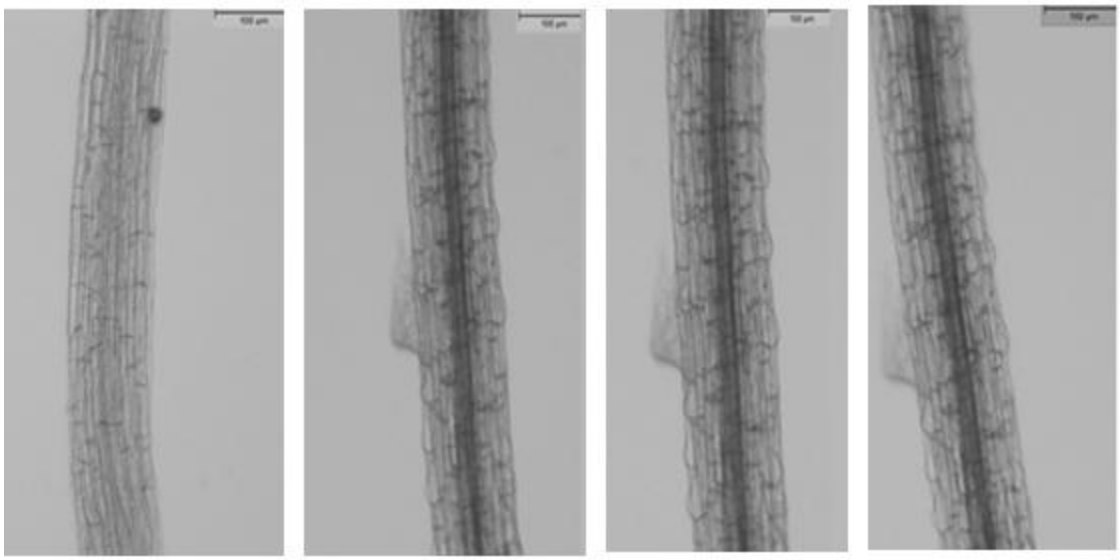


Figure 3.4: Lateral roots develop root hairs upon RSL4 induction.

Bright field images of the primary root of a 12 day old *rsl2 rsl4* double mutant seedling harbouring GR:RSL4, 24 hours after Dex treatment. Scale bars, A: 1mm, B, C: 200µm

3.3.3 Dex mediated induction of GR:RSL4 is fast

To determine the time needed for the induction of RSL4 upon Dex treatment I carried out a time course experiment. The H cells in the elongation zone of 12 day old GR:RSL4 *rsl2 rsl4* seedlings treated with Dex were imaged just after treatment (0^{th} hour) followed by every half an hour. Within 30 minutes of Dex treatment, small bulges characteristic of the early stage of root hair formation were seen on the H cells (Figure 3.5). On further incubation these bulges elongate by rapid tip growth giving rise to long root hairs (data not shown). These observations support the RSL4 expression data shown by Yi et al (2010) that RSL4 is present in the H cells just before hair growth and during growth itself (Yi et al., 2010). Together these results indicate that RSL4 mediated root hair development is a rapid process.



0 hour **30 mins** **1 hour** **2 hours**

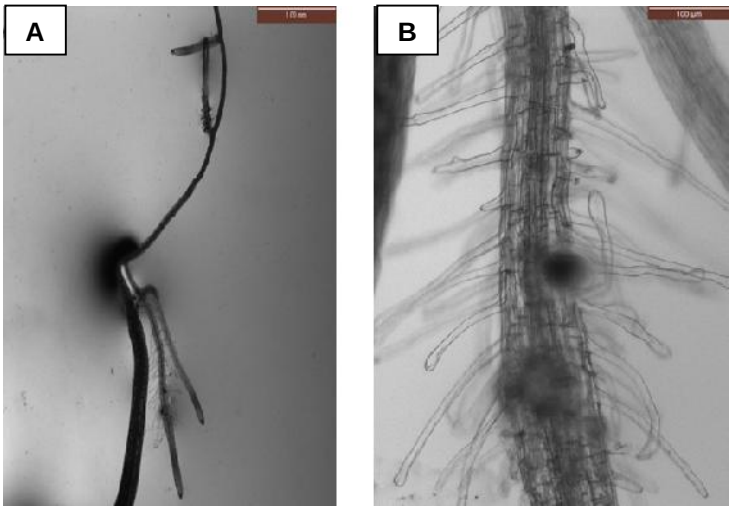
Figure 3.5: Root hair formation seen as early as 30 minutes.

Bright field images of a 12 day old *rsl2 rsl4* double mutant seedling* harbouring GR: RSL4, treated with 20µM Dex. Scale bar, 100 µm

3.3.4 Dex induces GR:RSL4 in young Arabidopsis seedlings

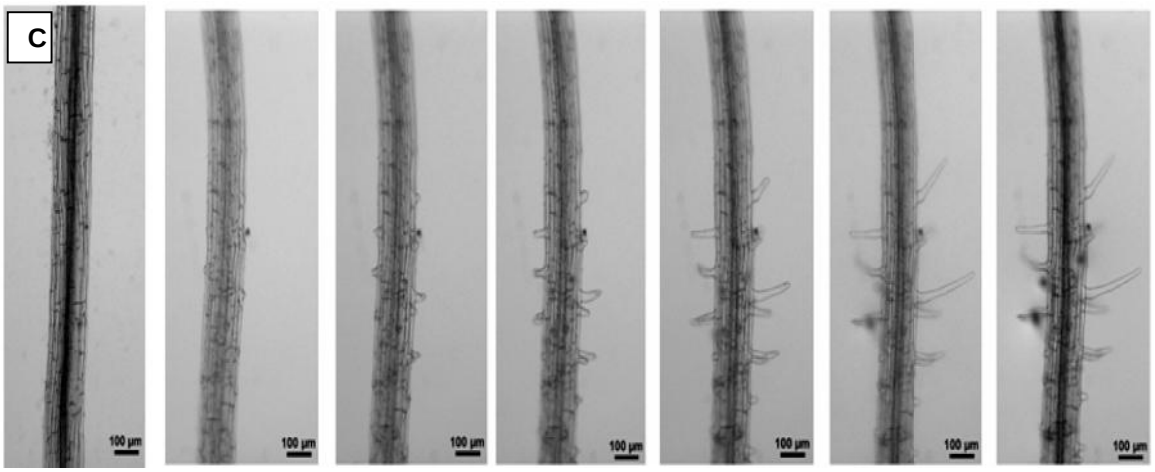
Given that RSL4 is an important regulator of the root hair growth process, I predicted that RSL4 would control root hair development in *Arabidopsis* from an early stage. To test this prediction I analysed the effects of RSL4 activation on the development of root hairs in the GR:RSL4 *rsl2 rsl4* seedlings of different ages ranging from 6-11 days after germination. The youngest seedlings used were 6 days old after germination, since by this stage most seedlings showed well-developed primary roots and not many lateral roots. Furthermore since the roots had to be harvested to generate material for the microarray analysis (explained in Chapter 4), seedlings younger than 6 days were not used. 6 day old seedlings incubated in MS liquid medium supplemented with 20 μM Dex were also able to develop root hairs both in the primary and lateral roots (Figure 3.6). Additionally Dex treatment was also able to rescue the root hair phenotype of 7-11 day old GR:RSL4 *rsl2 rsl4* seedlings. However, the excision of primary roots was easier in the young 6 day old seedling hence I used roots from these 6 day old GR:RSL4 *rsl2 rsl4* seedlings both without (Mock) and with Dex treatment for my microarray studies to identify the downstream targets of RSL4.

Lateral root:



24 hours

Primary root:



0 hour **3 hours** **4 hours** **6 hours** **8 hours** **13 hours** **23 hours**

Figure 3.6: Root hair formation is seen in both the primary and lateral roots of young (6 day old) GR:RSL4 rsl2 rsl4 seedlings.

Bright field images of GR:RSL4 *rsl2 rsl4* seedlings incubated for 24 hours in 20µM Dex. Scale bar, A: 1mm, B and C: 100 µm

3.3.5 Low concentrations of Dex are sufficient to induce RSL4

To determine the concentration of Dex required for the induction of RSL4 activity, I treated the 6 day old GR:RSL4 *rsl2 rsl4* seedlings with different concentrations i.e 5 µM, 10 µM, 15 µM and 20 µM of Dex. Root hairs initiated in the otherwise hairless 6 day old GR: RSL4 *rsl2 rsl4* seedlings when incubated in 5µM Dex to 20µM Dex concentrations, although with varying density. While at lower concentrations fewer and shorter hairs developed, at increased Dex concentrations all the H cells gave rise to root hairs that were longer than those incubated in low concentrations of Dex (Figure 3.7). However prolonged incubation in low concentrations of Dex led to the development of elongated root hairs.

Taken together these results indicate that the GR:RSL4 inducible expression system that I developed is sensitive to low concentrations of Dex and would serve as an efficient tool to identify the downstream targets of RSL4. In addition these data confirm the important role of RSL4 during root hair growth.

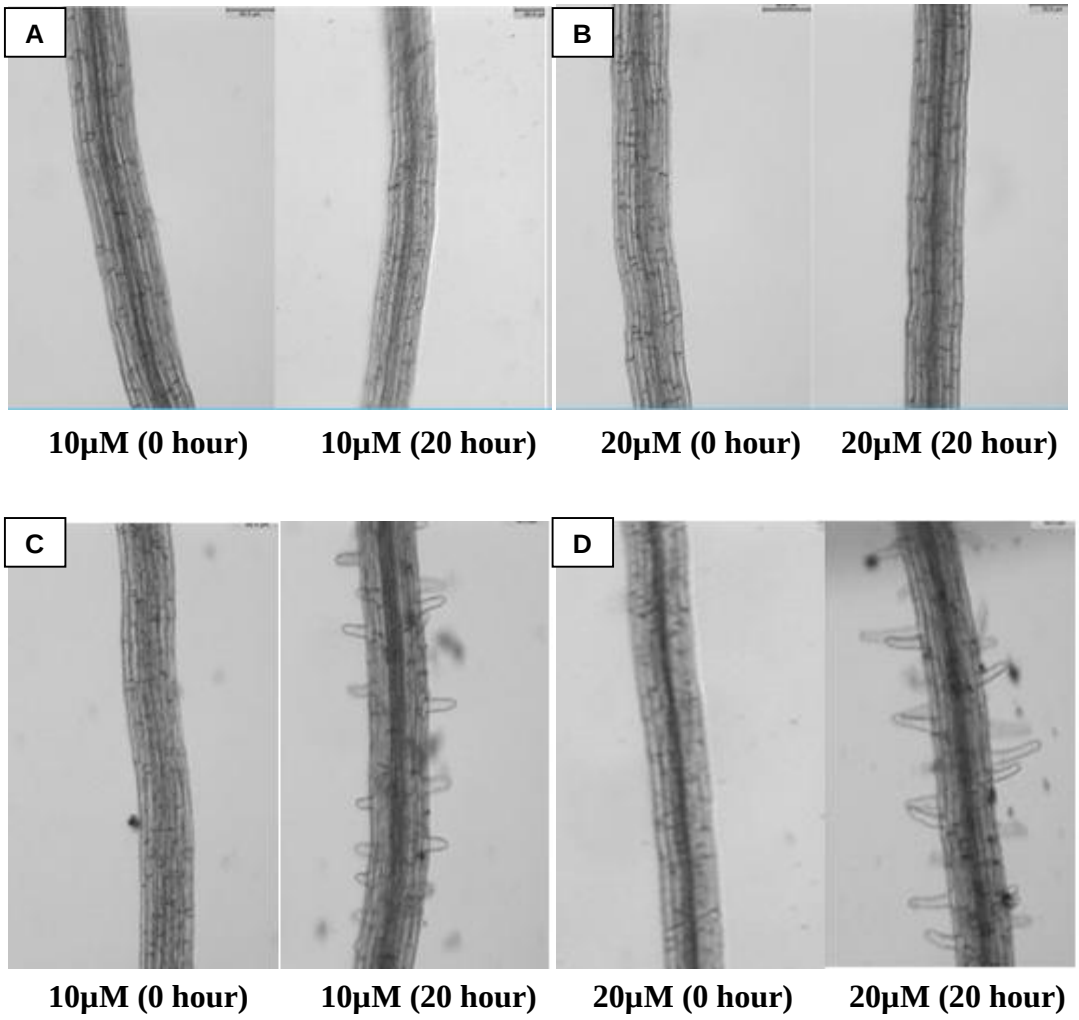


Figure 3.7: Root hair growth is induced even by reducing the initial concentration (20µM) of Dexamethasone (Dex) by half (10µM).

A, B: Bright field images of 6 day old *rsl2 rsl4* double mutant seedlings treated with MS liquid medium containing 20 µM, 10µM Dex. **C, D:** Bright field images of 6 day old *rsl2 rsl4* double mutant seedlings harbouring GR:RSL4 treated with MS liquid medium containing 20 µM Dex, 10µM Dex. Scale bar, 50 µm

3.4 Discussion:

3.4.1. RHD6–RSL4 transcriptional regulatory pathway underlies root hair development

The bHLH transcription factors, RHD6 and RSL4 control the initiation and elongation of root hairs respectively. Expression studies along with the phenotypic characterisation of the root hairs helped determine their essential role during root hair (H) cell development. *rhd6* and *rsl4* mutant plants developed fewer and shorter hairs (Masucci and Schiefelbein, 1994; Yi et al., 2010). Using a Dex inducible system *RSL4* was found to be induced only upon the activation of RHD6 in the mutant plants harbouring a glucocorticoid-inducible version of *RHD6* (*GR:RHD6*). The expression of *RSL4* was unchanged even upon the addition of cycloheximide (Cyc), a protein synthesis inhibitor indicating that RSL4 functions as an immediate target of RHD6 during root hair development. Based on all these observations it was concluded that the RHD6-RSL4 transcriptional network controls root hair development in a coordinated manner. Further characterisation of this pathway would help elucidate the regulatory cascade controlling the development of the H cell.

RSL4 being a bHLH transcription factor it is likely that it controls H cell development by regulating the expression of genes involved in cell growth processes. Preliminary microarray studies done by comparing the transcriptomes of the wild type, *rsl4* mutant and *CaMV 35S*:*RSL4* roots enabled the identification of 83 genes with reduced expression in the *rsl4* mutants and an increased expression in the *CaMV 35S:RSL4* plants. These genes were found to encode proteins functional during essential processes such as endomembrane trafficking and cell wall modification (Yi et al., 2010). This suggests that RSL4 controls root hair development by positively

regulating genes involved in growth processes. In order to validate the downstream targets identified via differential expression and also to identify the direct targets of RSL4, I adopted the glucocorticoid inducible expression system.

3.4.2 Glucocorticoid inducible system facilitates the temporal regulation of the RSL4 gene

Identification of the downstream target genes of several transcription factors has been enabled by the fusion of the GR receptor to the transcription factors (Sablowski and Meyerowitz, 1998; Levesque et al., 2006). In order to observe the changes in gene expression upon modulation of RSL4; I generated *rsl2 rsl4* mutant plants with *RSL4* fused to the glucocorticoid receptor (*GR*) under the control of the native *RSL4* promoter. *rsl2 rsl4* mutants harbouring GR:RSL4 rescued the development of root hairs only upon treatment with Dex verifying the role of RSL4 as a key regulator in root hair growth. This root hair development in the otherwise hairless *rsl2 rsl4* mutants was observed even after varying the concentrations of Dex from 5µM to 20µM concentration. This indicates that the GR: RSL4 system that I developed is an efficient system that is able to induce root hair formation even at lower concentrations of Dex.

All these experiments were conducted in older 12 day old seedlings. But in *Arabidopsis* once germination is synchronised a sturdy primary root is seen as early as in 6 day old seedlings. RSL4 being a key regulator of root hair development, Dex treatment of these younger 6 day old GR:RSL4 seedlings was tested to check if RSL4 induction was able to promote root hair development in younger seedlings. Indeed, Dex induction of RSL4 was able to induce root hair development in the young primary root

and in the lateral roots implying that RSL4 must be present early during the development of root hairs.

3.4.3 RSL4 regulates both the initiation and elongation of root hairs

Root hair cell morphogenesis comprises of three distinct stages namely: initiation, slow elongation and rapid tip growth (Dolan et al., 1994). Bibikova et al (1998) showed that the root hair initiation is intimately associated with the localized acidification of the cell wall at the site of initiation. This decrease in pH (4.5) marks the initial 30 minutes of hair initiation that is characterized by the bulging of the cell wall. Following initiation the hair undergoes tip growth during which the wall pH increases (pH6) (Bibikova et al., 1998). By modulating the RSL4 transcription factor using the GR inducible system, I found that upon activation, RSL4 mediates the bulging of the cell wall which is characteristic of the hair initiation stage. This phase can be clearly observed after 30minutes of Dex treatment further supporting Bibikova's finding that the initial half hour of cell wall acidification defines the start of root hair growth and RSL4 plays an essential role during this stage.

Subsequent to the initiation the root hairs undergo slow tip growth which lasts for approximately 2.5-3 hours. This slow growth phase is soon replaced by a rapid elongation phase when the hairs grow more quickly to give rise to long hairs similar to that of wild type, after 24 hours of RSL4 induction (Figure 3.2). Additionally these results extend the data described by Yi et al (2010) which showed that upon fusion with GFP, RSL4 is expressed in hair cells in the elongation zone just before root hairs initiated and during hair growth (Yi et al., 2010). Together these observations implicate

the function of RSL4 in regulating root hair development right from the early stages of root hair initiation to the later stages of root hair growth.

On the whole the GR:RSL4 inducible system that I developed serves as a powerful tool that could facilitate a clear understanding of the regulatory mechanisms underlying root hair cell growth. By modulating RSL4 the relative expression of the genes controlled by RSL4 during root hair growth can be determined. The genes identified as part of the transcriptomic analysis would in turn serve as a cue to trace the RHD6-RSL4 transcriptional pathway further.

Chapter 4:
RESULTS

4. Identification and characterization of genes differentially expressed upon RSL4 induction.

4.1 Abstract:

Root hair (H) cell development serves as a model for studying the genetic regulatory mechanisms underlying plant cell morphogenesis. A class of important regulatory genes namely *RHD6* and *RSL4* belonging to the bHLH subfamily VIIIc have been found to temporally control root hair initiation and tip growth respectively. RSL4 was identified as an immediate target of RHD6 and functions as a key regulator of root hair growth. Preliminary microarray studies suggest that RSL4 controls root hair elongation by regulating the expression of genes that encode proteins that are involved in cell growth processes. Identification of the target genes of RSL4 would help elucidate the molecular machinery underlying H cell development. Here I performed genome wide transcriptional analyses after modulating RSL4 using a glucocorticoid inducible expression system. Using this method, I identified with high statistical significance (p value <=0.001) thirty four genes positively regulated by RSL4. Additionally even in the presence of cycloheximide (Cyc), a protein synthesis inhibitor the expression of these genes was unaffected indicating that they could be directly regulated by RSL4. As part of preliminary analysis a subset of nine genes were screened for defects in root hair development. Mutations in four genes: *AT1G30870* (*PRX7*), *AT5G03540* (*EXO 70A1*), *AT1G22620*(*SAC1)* and *AT1G35670*(*CPK11*) caused defects in root hair morphogenesis. Proteins encoded by these genes regulate the modification of the cell wall, vesicle trafficking, the organization of the cytoskeleton and signal transduction emphasising the importance of these processes during root hair tip growth.

4.2 Introduction:

RHD6 and RSL4 transcription factors regulate root hair development by controlling the initiation and elongation of the root hair cell. However little is known about the mechanism by which RSL4 functions to enable root hair elongation. Several approaches have been used in the past to identify root hair morphogenetic genes including mutant screening (Schiefelbein and Somerville, 1990) and microarray studies analysing the root hair cell transcriptome (Jones et al., 2006).

The present study was used to gain further insight into the role of RSL4 in regulating the H cell development. Earlier using a glucocorticoid inducible expression system, *RSL4* was identified as an immediate target of RHD6 (Yi et al., 2010). Here I adopted a similar approach and expressed an inducible version of RSL4 in the *rsl2 rsl4* mutant background in order to identify the target genes regulated by RSL4. Furthermore, concurrent treatment with Dex and the protein synthesis inhibitor cycloheximide (Cyc) was used to identify the immediate target genes that acquire regulatory cues from RSL4 to enable root hair growth.

4.3 Results:

4.3.1. Simultaneous treatment with Dex and Cyc can be used to identify potential RSL4 direct target genes

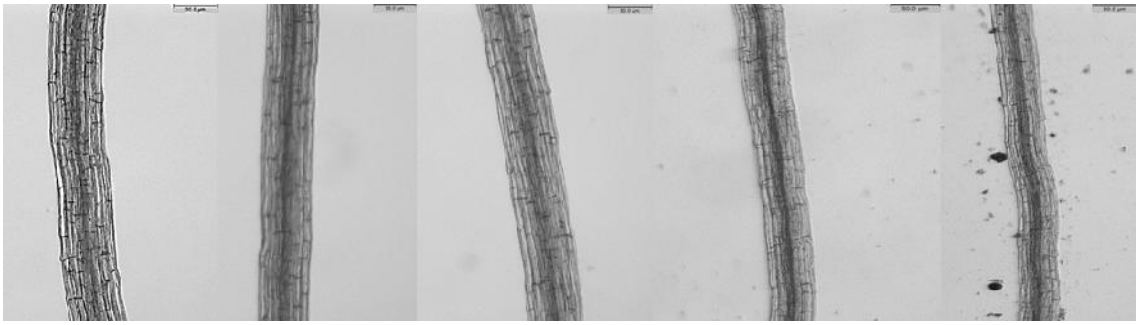
In order to identify the RSL4 direct targets, I generated a glucocorticoid inducible form of RSL4 (as described in detail in Chapter 3). The *GR:RSL4* construct was transformed into the *rsl2 rsl4* mutant background which is root hairless. In the absence of the Dex induction the *rsl2 rsl4* plants harbouring GR:RSL4 remained hairless identical to the *rsl2 rsl4* double mutant plants. However upon the addition of Dex, I observed root hair growth indicating the rescue of the RSL4 activity. RSL4 being a key regulator of root hair growth, restored the root hair growth in the *rsl2 rsl4* plants harbouring GR:RSL4.

As discussed in the previous chapter, root hair development in the form of an elongated bulge was clearly seen 4 hours after transfer into a medium supplemented with 20 µM Dexamethasone (Dex) in 6 day old *GR:RSL4 rsl2 rsl4* seedlings. This time point was chosen to harvest the root tissue for the microarray analysis where the transcriptomes of Mock treated and Dex treated roots were compared.

While the induction of RSL4 by Dex would help identify all the target genes that are regulated by RSL4, the identification of the specific direct RSL4 targets would help trace the precise RHD6-RSL4 transcriptional regulatory pathway controlling root hair growth. Concurrent treatment of the GR inducible plants with Dex and cycloheximide (Cyc) has been used to identify the direct targets as seen in the case of floral development transcription factors (Sablowski and Meyerowitz, 1998). Cyc is a protein synthesis inhibitor that presumably allows the transcription of the immediate target genes while blocking the indirect target genes by inhibiting the translation of intermediary factors. Here as a first step, I observed the effects of the Dex and Cyc

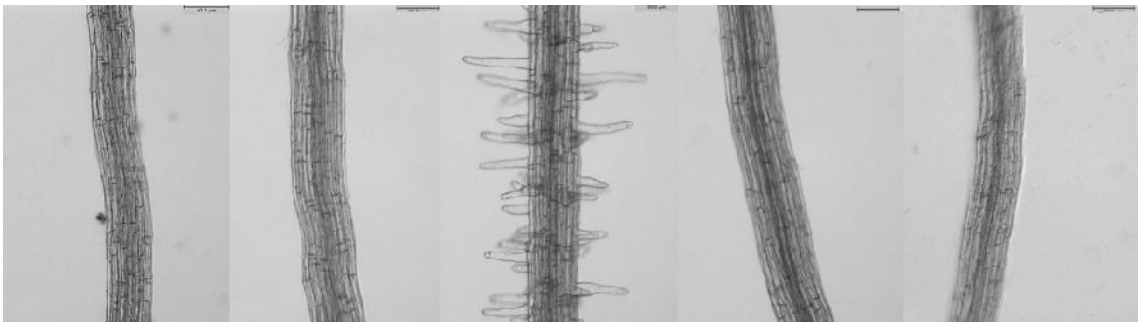
treatment on the root hair phenotype of the GR:RSL4 inducible plants. Treatments were done using 12 day old GR:RSL4 *rsl2 rsl4* seedlings. Upon simultaneous treatment with Dex and Cyc the development of root hairs was completely inhibited indicating that Cyc prevents hair development by inhibiting the translation of downstream factors (Figure 4.1). However as Cyc allows the transcription of genes to occur, comparison of the transcriptional profiles of roots treated with Dex and Dex + Cyc would help identify the direct targets of RSL4.

0 hour:



Untreated **Mock** **Dex** **Cyc** **Dex + Cyc**

24 hours:



Untreated **Mock** **Dex** **Cyc** **Dex + Cyc**

Figure 4.1: Cycloheximide inhibits root hair formation.

Representative bright field images of 12 day old GR: RSL4 *rsl2 rsl4* seedlings under different treatments i.e 20µM Dex and 20µM Cyc. Scale bar, 50µm

Previously a microarray done in the lab had compared the transcriptional profiles of Col-0, *rsl4* and *35S:RSL4* to identify potential downstream targets of RSL4 (Yi et al., 2010). I picked two genes from this list namely *AT1G34510/PRX8,* and *AT4G30320* to check the effect of RSL4 induction by Dex on their expression levels by qPCR. I found that the expression levels of the two genes were up-regulated upon Dex treatment of the GR:RSL4 *rsl2 rsl4* seedlings (Figure 4.2). Additionally the effect of the simultaneous application of Dex and Cyc on the expression of these genes was tested. After 4 hours of treatment with Dex and Cyc, *AT1G34510/PRX8* did not show a change in gene expression when compared to that in the Dex treated seedlings suggesting that they could be directly regulated by RSL4 (Figure 4.2). On the other hand the expression level of *AT4G30320* after Cyc treatment was repressed indicating that *AT4G30320* could function further downstream in the RSL4 transcriptional pathway. Taken together I devised a system to identify the direct targets of RSL4.

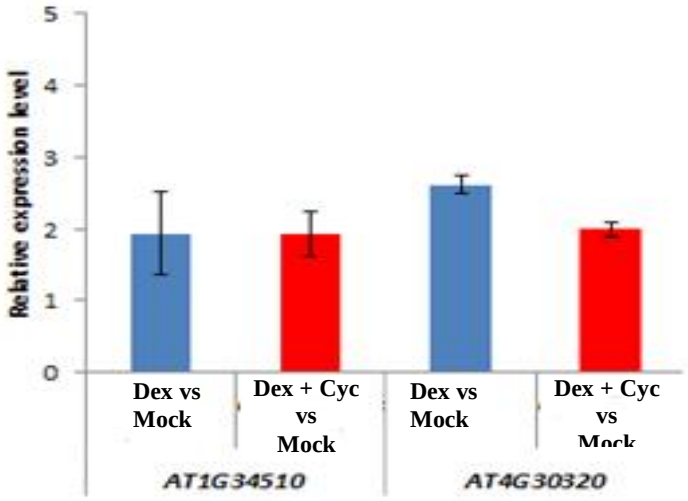


Figure 4.2: Relative expression levels of putative RSL4 target genes 4 hours after incubation with Dex, Dex + Cyc and Mock.

Bars indicate standard deviation

4.3.2 Microarray analysis coupled with the GR inducible expression system identified putative RSL4 targets

After modulating the RSL4 activity using different chemical treatments (Mock, Dex, Dex + Cyc), I first performed three independent genome wide transcriptional analyses. Each experiment constituted of a biological replica with three treatments. Affymetrix ATH1 arrays were used to determine the transcriptional profiles of the mRNA isolated from the root tips of the 6 day old GR:RSL4 *rsl2 rsl4* seedlings, 4 hours after incubation with a) Mock b) Dex and c) Dex + Cyc.

The microarray data sets were then analysed and the genes that were differentially expressed between the Mock, Dex and Dex + Cyc treatments were identified using the following criteria: equal to and more than two - fold change in expression level applying a stringent filter i.e P value <=0.001. 993 genes that were induced in Dex versus Mock samples were identified. Additionally on comparing the Dex + Cyc versus Mock samples, 255 genes were found to be up regulated. The two data sets (Dex vs Mock and Dex + Cyc vs Mock) showed an overlap of 128 genes indicating that these genes were significantly induced in both the Dex treated samples and in the Dex + Cyc treated samples (Figure 4.3A).

Further in order to identify the putative RSL4 immediate target genes among the 128 overlapping genes the relative expression levels of the genes was analysed (Figure 4.3B). 34 of these genes (27% of the total of selected genes) were found to have an unchanged expression level in both the Dex vs Mock and the Dex + Cyc vs Mock samples (Figure 4.4) (Genes with fold change values are listed in Appendix 9). These 34 genes whose transcription is unaffected by Cyc treatment could be potential direct targets of RSL4.

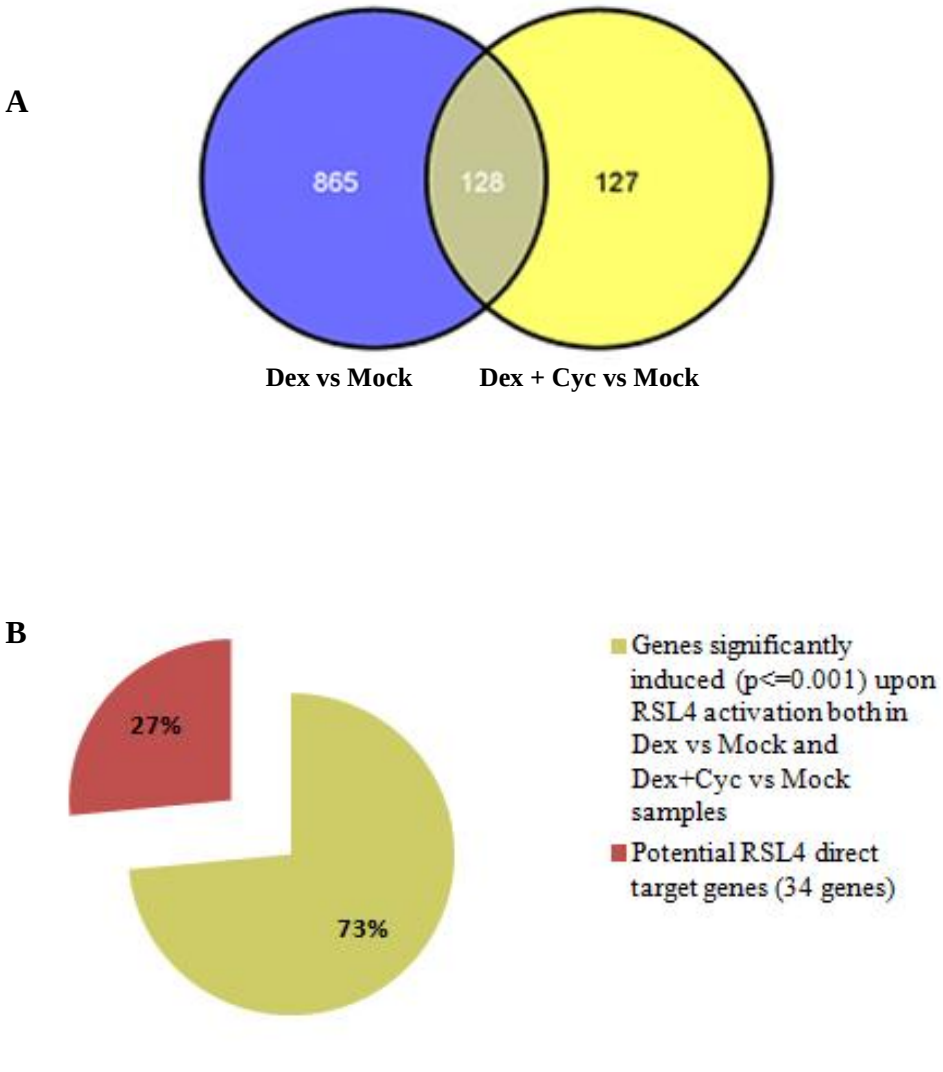


Figure 4.3: Thirty four potential RSL4 direct target genes have been identified.

A: Venn diagram of genes induced upon RSL4 activation under different treatments. Blue circle depicts genes induced in Dex vs Mock sample, Yellow circle represents the genes induced in Dex + Cyc vs Mock sample. The grey region represents the overlapping genes. **B:** Pie chart showing the percentage of putative RSL4 direct target genes i.e. 34 genes out of the 128 overlapping genes (statistical significance, p<=0.001).

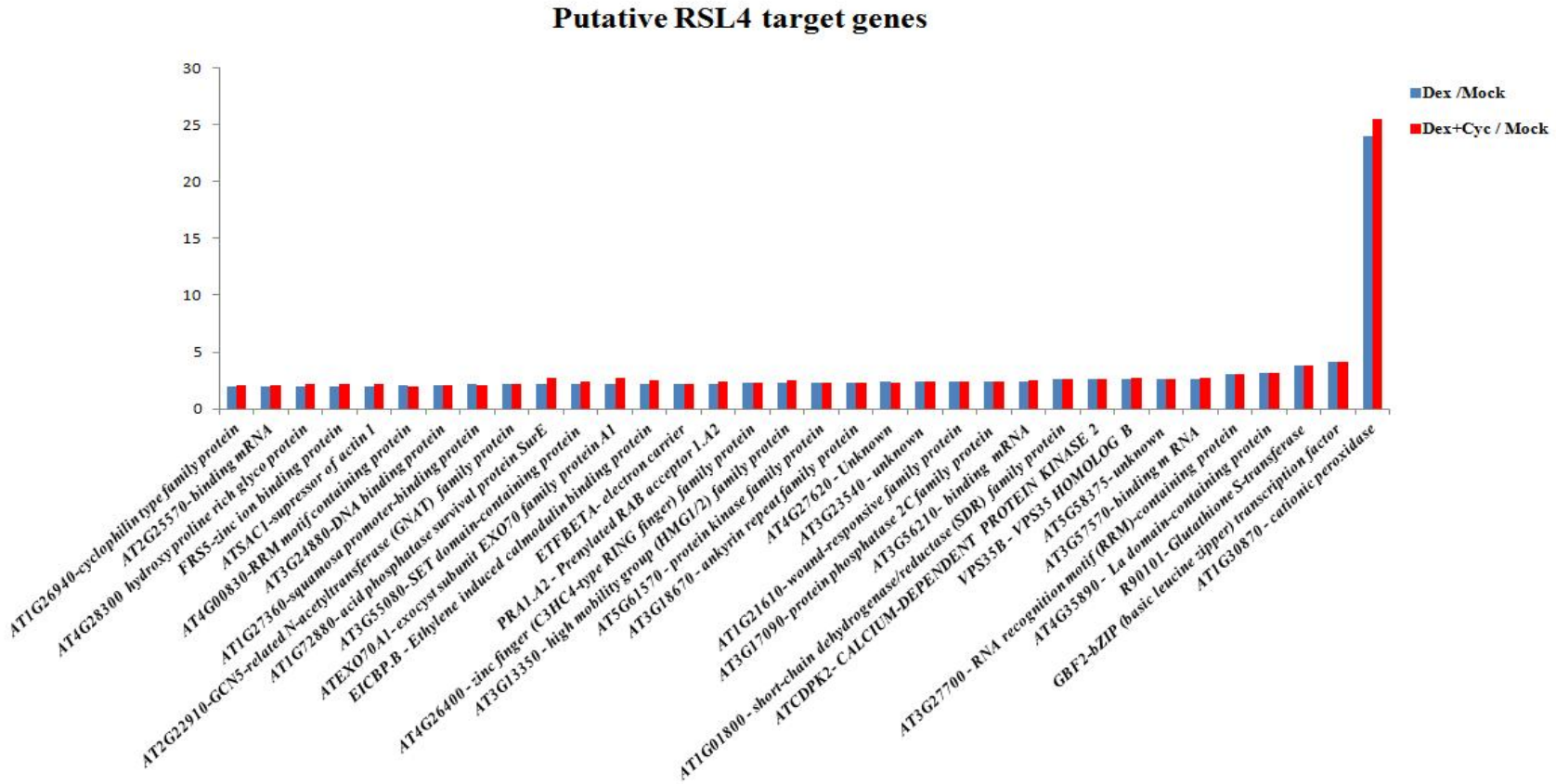


Figure 4.4: Bar graph of putative direct targets of RSL4.

Blue bar shows the relative gene expression upon RSL4 induction by Dex treatment. Red bar shows the relative gene expression after the simultaneous treatment with Dex and Cyc.

4.3.3 Potential RSL4 target gene mutants (prx 7, exo 70A1, sac 1 and cpk 11) develop aberrant root hair phenotypes

RSL4 being an important regulator of root hair growth, it can be predicted that its direct target genes would also function during root hair development. In order to test the role of the candidate target genes in root hair development I ordered T-DNA insertion lines for a subset of 9 genes from the list of 34 potential RSL4 direct target genes.

From a segregating T2 population, homozygous T-DNA insertion mutants were identified by genotyping (primers are listed in Appendix 3), and their root hair phenotypes were observed. Out of the 9 genes characterized the T-DNA mutants in four genes produced a short root hair phenotype. The genes and their mutant lines are as follows *AT1G30870/PRX7 (GK_476A09;prx7), AT5G03540/EXO70A1 (SALK_014826; exo70A1-1, SALK_135462; exo70A1-2), AT1G22620/SAC1 (SALK_083405; sac1),* and *AT1G35670/CPK11/CDPK2 (SALK_054495; cpk11*). The phenotypes observed were based on preliminary analysis from a segregating population (Table: 2). Further phenotypic characterization is necessary to confirm the results observed.

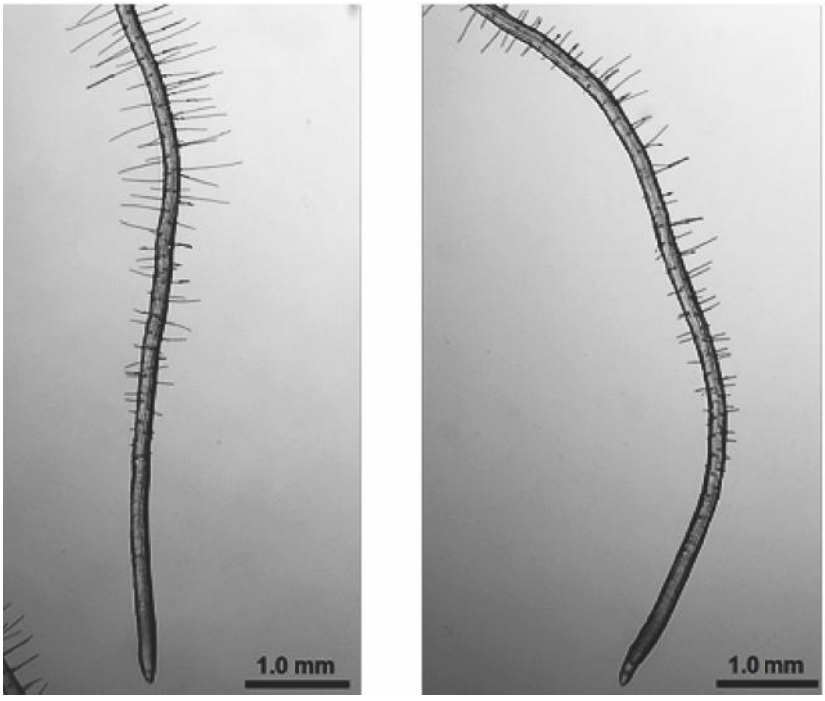
In *Arabidopsis, PEROXIDASE7 (PRX7)* encodes a class III peroxidase (Tognolli et al., 2002; Valerio et al., 2004). When compared to the wild type plants the *prx7* mutants developed shorter root hairs indicative of a role of *PRX7* in root hair elongation (Figure 4.5).

Similar to that reported by Synek et al (2006), *exo70A1* mutants had shorter roots and retarded lateral root development. EXO70A1 is a subunit of the octameric exocyst complex in *Arabidopsis* (Synek et al., 2006). I observed that the root hairs that developed in the *exo70A1* mutants were short as a result of defective tip elongation

(Figure 4.6). In several H cells the root hairs initiate only as bulges that fail to elongate indicating that the transition to tip growth is inhibited.

Defects in root hair development were also observed in the T-DNA insertion mutant of the *SUPRESSOR OF ACTIN 1 (SAC1*) gene. *sac1* mutants exhibit a decrease in root hair length when compared to the wild type seedling suggestive of a defect in root hair growth (Figure 4.7).

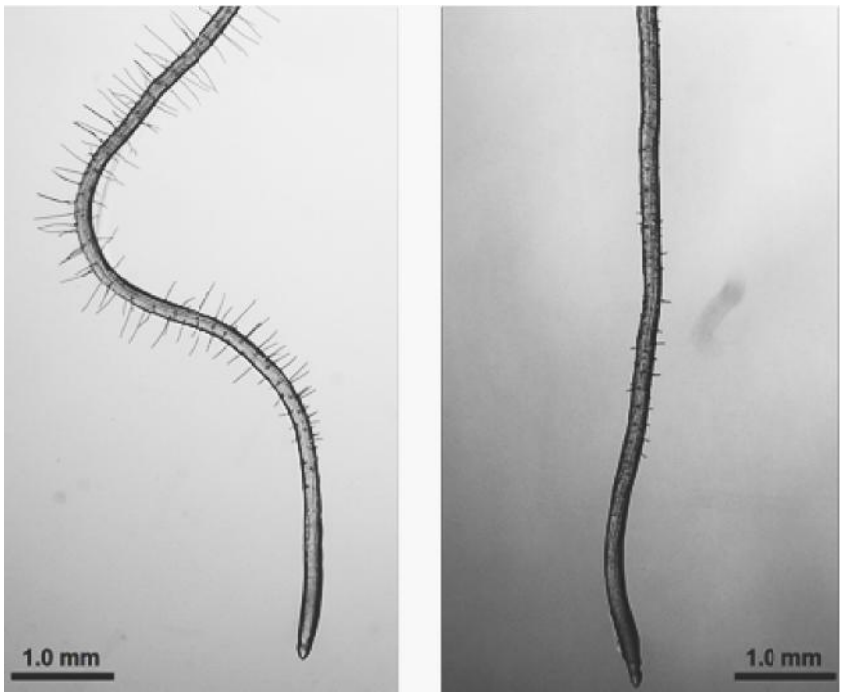
cpk11 mutants have been shown to develop longer roots in the presence of abscisic acid (ABA) (Zhu et al., 2007). *CPK11* encodes a calcium - dependent protein kinase (Harmon et al., 2001). When grown on Paul's media without ABA, I observed that these mutants developed roots similar in length to that of the wild type seedlings; however an alteration in root hair development was seen. The mutants develop root hairs that have reduced length when compared to the wild type seedlings (Figure 4.8). Taken together these results provide further evidence that *PRX7, EXO70A1, SAC1* and *CPK11* are direct targets of *RSL4* and are required for normal root hair growth in *Arabidopsis*.



Col-0 ***prx 7***

Figure 4.5: *prx 7* develops root hairs that are shorter than in Col-0.

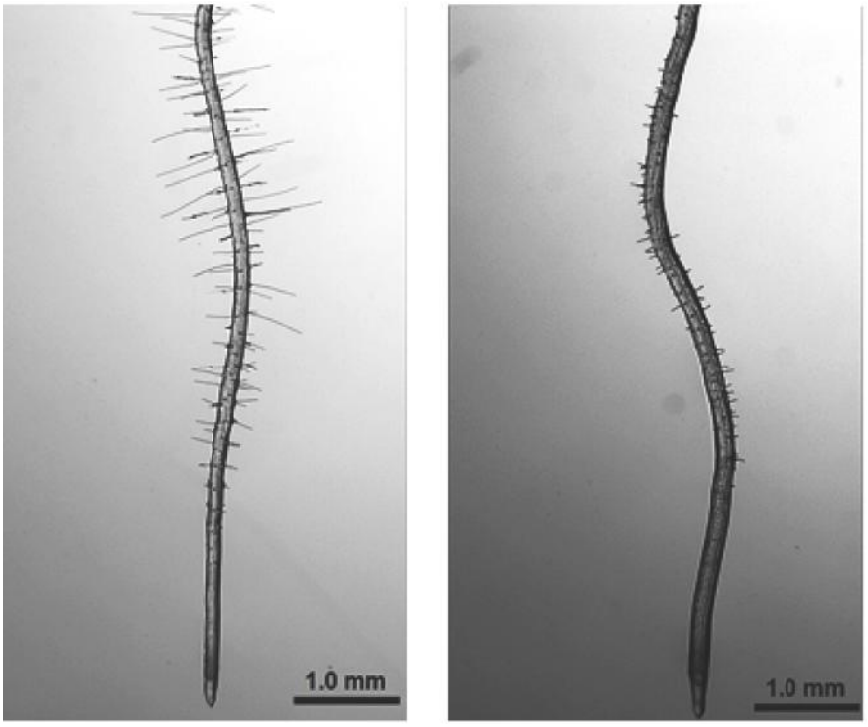
Representative bright field images were obtained from seven day old wild type and *prx 7* mutant. Scale bar, 1mm



Col-0 ***exo70A1***

Figure 4.6: *exo70A1* mutant seedlings exhibit defective root hair elongation.

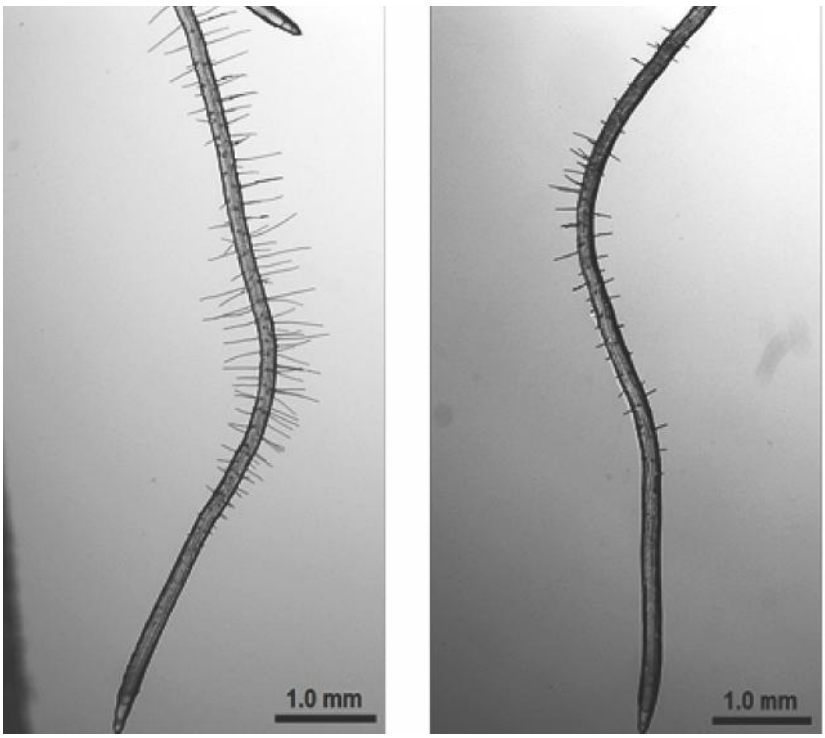
Representative bright field images were obtained from seven day old wild type and e*xo 70 A1* mutants. Scale bar, 1mm



Col-0 ***sac1***

Figure 4.7: s*ac 1* mutants are characterised by a defect in root hair growth.

Representative bright field images were obtained from seven day old wild type and *sac1* mutants. Scale bar, 1mm



Col-0 ***cpk11***

Figure 4.8: *cpk 11* mutants develop shorter root hairs when compared to Col-0.

Representative bright field images were obtained from seven day old wild type and *cpk11* mutant. Scale bar 1mm.

4.4 Discussion:

RSL4 is a bHLH transcription factor of the RSL family of proteins and plays an essential role in root hair growth in *Arabidopsis* (Yi et al., 2010). Here I have used a microarray based approach to identify the downstream targets of RSL4 in order to outline the RHD6-RSL4 mediated transcriptional network underlying root hair cell development. As a result of the transcriptional profile analysis done after modulating the activity of RSL4, I identified 34 putative RSL4 direct targets. The expression of these putative target genes was induced upon RSL4 activation by Dex and this induction remained unchanged even upon treatment with Cyc, a protein synthesis inhibitor. Based on this data it can be predicted that the 34 identified genes could be directly regulated by RSL4. However, further analysis may be necessary to substantiate their role in the RSL4 mediated root hair cell growth. Hence I selected a subset of nine genes, (refer Appendix 9) based on root specific expression pattern [as previously reported by analysis of several microarray data, tool used - the electronic Fluorescent Pictograph (eFP) Browser, available at http://www.bar.utoronto.ca/] (Winter et al., 2007) and for which the T-DNA insertion lines were readily available and screened them for root hair phenotypes. Four out of the nine chosen genes exhibited an alteration in root hair growth suggesting a possible function of these genes in the H cell development.

4.4.1 Putative role of the RSL4 target genes in root hair development

In my mutant analysis four T-DNA insertion mutants namely *prx7, exo70A1, sac1*, and *cpk11* showed an altered root hair phenotype (Table 2). These results serve as preliminary evidence for the role of *PRX7, CPK11, SAC1* and *EXO70A1* in H cell

development possibly through the RHD6-RSL4 pathway. Here I will discuss the potential roles of the selected four genes in the RSL4 regulated growth of root hairs.

Table 2: Putative RSL4 target genes exhibiting an aberrant root hair phenotype.

No	Fold Change		Gene	Root hair phenotype
	Dex vs Mock	**Dex + Cyc vs Mock**		
1.	2.03	2.18	*AT1G22620/SAC1* (*SUPPRESSOR OF ACTIN 1*) Phosphatidylinositol - 4,5-bisphosphate 5-phosphatase	Short root hairs
2.	2.24	2.71	*AT5G03540/EXO70A1* (*EXOCYST 70A1*) Exocyst subunit	Root hairs with defective elongation
3.	2.61	2.65	*AT1G35670/ CPK11/ CDPK2 (CALCIUM DEPENDENT PROTEIN KINASE 11)* Protein kinase	Short root hairs
4.	23.96	25.48	*AT1G30870/PRX 7 (PEROXIDASE 7)* Cationic peroxidise	Short root hairs

4.4.2 PRX 7 functions during root hair cell elongation

PRX7 encodes a class III peroxidase. In *Arabidopsis* the class III peroxidase (PRX) is a multigene family that comprises of 73 genes (Tognolli et al., 2002; Valerio et al., 2004). Due to the large gene family often the mutations in one *PRX* gene does not reveal any dramatic mutant phenotype. This could possibly be due to gene redundancy; as seen in the case of *PRX8* and *PRX60*, explained in Chapter 5. *PRX8* and *PRX60* were identified as potential targets of RSL4 (Yi et al., 2010), however the loss of function mutants of these genes did not exhibit any aberrant root hair phenotypes.

My microarray analysis identified another peroxidase gene, *PRX7* that is induced upon RSL4 activation by Dex. Further this induction remained unchanged even upon concurrent treatment with Dex and Cyc, suggestive of the direct regulation by RSL4. Further evidence for the role of PRX7 in the root hair development was obtained from the phenotypic characterisation of the loss of function mutant; *prx7* developed short root hairs when compared to the wild type.

Root hair growth is characterised by the modification of the plant cell wall. Plant cell walls are dynamic compartments that play an essential role in providing shape and support to the cells (Fry, 2004; Galway, 2006). Several enzymes such as the class III peroxidases have been implicated in the cleavage and reassembly of the cell wall components. Peroxidases through their two catalytic cycles: peroxidative and hydroxylic enable changes in the internal structure of the cell wall. By regulating the availability of H_2O_2 which is a prerequisite for the cross linking of several compounds (eg: extensins), peroxidases mediate wall rigidification. Additionally by generating reactive oxygen species (ROS), peroxidases enable wall loosening (Passardi et al., 2004). *PRX33* and *PRX34* have been shown to be involved in root elongation by

promoting cell wall loosening (Passardi et al., 2006). Together these results suggest a role for PRX7 in root hair growth either by the way of promoting cell wall rigidification or wall loosening.

4.4.3 EXO70A1 is required for tip growth of root hairs

Root hair development can be divided into three phases namely initiation and bulge formation followed by transition to slow tip elongation that eventually leads to rapid tip growth (Dolan et al., 1994). Genetic analysis led to the characterisation of root hair defective mutants such as *exo70A1*. Unlike wild type plants mutants of the *EXO70A1* gene showed abnormal root hair development. These mutants developed bulges which failed to elongate, indicative of a defect in transition to tip growth. *EXO70A1* encodes an exocyst subunit in *Arabidopsis* (Synek et al., 2006). The exocyst is an octameric secretory vesicle tethering complex that spatially regulates exocytosis which is important for cellular functions such as the incorporation of proteins on to the growing cell membranes (Li et al., 2010).

Further Kulich et al (2010) showed that *EXO70A1* is essential for the polarized seed coat pectin delivery providing evidence for the role of the EXO70A1 in the transport of cell wall materials during morphogenesis (Kulich et al., 2010). Together these observations indicate that EXO70A1 is essential for the vesicle trafficking of membrane proteins during the tip growth of H cells. Further my microarray data also revealed that EXO70A1 could function during root hair tip growth by being regulated by RSL4.

4.4.4 SAC1 regulates cell wall synthesis and actin organization

SAC1 (*SUPPRESSOR OF ACTIN 1*) was identified as a potential direct target of RSL4. *SAC1* encodes a phosphoinositide (PI) phosphatase. SAC domain phosphatases are a novel group of phosphatases that were first identified in yeast and animals and have been shown to hydrolyse phosphates at several positions of the inositol head group of PI (Hughes et al., 2000). In *Arabidopsis* nine suppressor of actin (SAC) domain PI phosphatase-like proteins (SAC1-9) have been identified.

During development the lipid phosphatases function to regulate the synthesis and turnover of phosphoinositides (PI). PIs are a group of phospholipids that regulate cellular processes such as signal transduction, vesicle trafficking and the organization of the actin cytoskeleton (Zhong et al., 2005). The identification of the *FRAGILE FIBER 3 (FRA3*) gene which encodes a Type II inositol polyphosphate phosphatase was pivotal in elucidating the role of PI phosphates during plant cell development. The *fra3* mutants exhibited cell wall defects and abnormal actin organization in fibre cells (Zhong et al., 2004). Similarly screening for other defective fiber mutants, led to the identification of the *fra7* mutant that exhibited an alteration in the actin cytoskeletal organization along with a decrease in the fiber cell elongation. The gene responsible for this mutation was identified as *SAC1* (Zhong et al., 2005). Together these observations indicate that in plant cells PI phosphatases such as FRA3 and SAC1 function during growth by regulating the actin cytoskeleton and cell wall synthesis.

4.4.5 Mutant analysis reveals the role of SAC1 in root hair development

My microarray data showed that the gene expression of *SAC1* is regulated by RSL4 suggesting that this PI phosphatase could also function during H cell development. In

order to determine if *SAC1* has a role in the *RSL4* mediated root hair cell growth I observed the root hair phenotype of the *sac1* mutant. *sac1* has a root hair elongation defect. While the root hairs in the mutant initiate normally most hairs fail to elongate and remain as bulges on the surface of the root. Some hairs that do elongate develop short root hairs when compared to the wild type.

The absence of an aberrant root hair phenotype in the fra7 allele as observed by Zhong et al (2005), could be because the *fra 7* mutation resulted only in the truncation of the C terminus of *SAC1* causing no effect to the phosphatase activity (Zhong et al., 2005). Whereas the sac1 allele used in my study had the mutation in the 5'UTR of *SAC1* producing a null transcript and thereby rendering it non-functional. The metabolism of PIs involve the cyclic process of phosphorylation and dephosphorylation catalysed by PI kinases and PI phosphatases respectively leading to their distinct subcellular localization (Matteis and Godi, 2004). *SAC1* was found to exhibit phosphatase activity; possibly dephosphorylating PtdIns(3,5)P2 to phosphatidylinositol 3-phosphate [PtdIns(3)P]. In the reverse reaction PtdIns(3)P is phosphorylated by a PtdIns3P 5-kinase (*FAB1)*, back to PtdIns(3,5)P2. Interestingly, the overexpression and mutant lines of the *FAB1* gene exhibited short root hairs indicative of their essential role in root hair development (Hirano et al., 2011). VPS 34 is a class III PI3-kinase that synthesizes PtdIns(3)P. Inhibition of VPS34 activity resulted in shorter root hairs with slower growth rates when compared to the wild type plants (Lee et al., 2008). On the whole these results suggest the importance of the PtdIns(3)P pathway in regulating root hair cell growth. SAC1 being part of this pathway we could postulate that it controls root hair growth as a result of direct regulation by the RSL4 transcription factor.

4.4.6 CPK11 is a putative RSL4 target

In addition to *PRX7, EXO70A1* and *SAC1*, *CPK11* was identified as a potential direct target of RSL4 using an inducible RSL4 protein driven from its native promoter. CPK11 encodes a calcium-dependent protein kinase that has been shown to regulate the abscisic acid signalling pathways in *Arabidopsis* (Zhu et al., 2007). Further based on my microarray studies, I characterised the root hair phenotype of the loss of function mutant of *CPK11* to understand the role of this protein kinase in the RSL4 mediated root hair growth. The *cpk11* mutant produced shorter root hairs supporting the function of *CPK11* in regulating root hair cell development.

Calcium dependent protein kinases (CPKs/CDPKs) are characterised by the presence of C-terminal calmodulin-like regulatory domain with four calcium binding EF hands and their activity is regulated by Ca^{2+} signals (Patil et al., 1995; Sanders et al., 1999). Further the maintenance of tip focussed cytoplasmic calcium ($Ca^{2+)}$ is indispensable for root hair growth. A higher concentration of cytoplasmic Ca^{2+} is seen at the growing tip region when compared to the rest of the hair cell (Bibikova et al., 1997). These results suggest that CPK11 could be a member of the calcium signalling pathway regulating RSL4 mediated root hair elongation.

Together my data revealed that RSL4 mediates root hair cell growth in *Arabidopsis* by regulating its potential target genes such as *PRX7, EXO70A1, SAC1* and *CPK11*.

4.5 Future prospects

The genome-wide transcriptional analyses done after modulating the RSL4 activity provides a powerful method of identifying its downstream genes. The presence of a root hair phenotype in 4 out of the 9 potential RSL4 target genes chosen for the preliminary functional characterisation highlights the effectiveness of the screen done using the glucocorticoid inducible version of RSL4.

Studies in our lab have shown that the *RSL* family of genes are present across plant species like mosses (Menand et al., 2007). This indicates that genetic pathways are conserved during evolution. Hence understanding the core machinery that controls H cell development will also serve as a blue print that could be used to study similar growth mechanisms in other plants and also facilitate genetic manipulations for agricultural benefits.

However, further confirmatory experiments would be necessary to narrow down on specific target genes that could be functional in the RHD6-RSL4 transcriptional pathway. Certain genes may be expressed only in small subset of cells at a particular time point indicating that there are chances of detecting false positives. The solution to this problem would be to generate other complementary transcriptional profile datasets, such as the transcriptional profiles of mRNA extracted from the root hair differentiation zone of the loss of function mutant (*rsl4*) and overexpression (*35S:RSL4*) seedlings grown under similar growth conditions as used during the GR experiment. Consistency seen from such independent experiments would minimise the detection of false positives. Further, the statistical information obtained from several independent experiments can be combined to identify statistically significant RSL4 target genes.

Following the determination of potential direct RSL4 target genes a chromatin immunoprecipitation – based microarray (ChIP-chip) experiment needs to be performed This method has been used efficiently to identify the genome wide location of the direct targets of transcription factors such as SHORTROOT (SHR) (Cui et al., 2011). A similar approach can be useful to assay the binding of RSL4 to the promoter region of its target genes. Further the role of the identified RSL4 targets in root hair development can be confirmed by observing their expression patterns using in situ hybridization and by using reporters such as fluorescent proteins example: green fluorescent protein (GFP).

Chapter 5:
RESULTS

5. Characterization of differentially expressed genes in the gain and loss of function mutants of *RSL4* suggests functional redundancy

5.1 Abstract:

ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4) is a key regulator of post mitotic root hair cell growth. Microarray experiments done to identify genes differentially expressed in *Arabidopsis* wild-type, *rsl4* mutant and *CaMV 35S*:*RSL4* roots, identified 83 genes with both decreased transcript levels in *rsl4* and increased transcript levels in *CaMV 35S*:*RSL4* transformed plants. 20% of these differentially expressed genes have been previously hypothesized to be functional during cell growth suggesting that they could function as downstream targets of *RSL4* during H cell growth. Hence I used this data to conduct an independent analysis to identify the target genes regulated by RSL4. A subset of these potential RSL4 target genes (10 genes) that exhibited a change in gene expression both in the *rsl4* mutant and the *RSL4* overexpressor line were selected from the overall list for functional characterisation. The genes selected primarily encode cell wall structural proteins and wall modifying enzymes. Further some of the putative targets also encode signal transduction molecules and pathogenesis related proteins. Phenotypic characterization of the loss of function mutants and the overexpression lines of the selected ten genes showed no alteration in root hair development. The absence of any distinct root hair phenotype can be attributed to the high functional redundancy among these genes indicating that these genes are not as crucial as RSL4 during root hair development. Further these results also suggest that these genes have no role in root hair development.

5.2 Introduction:

In *Arabidopsis*, ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4) was identified as a key regulator of root hair cell growth. The loss of function mutant (*rsl4*) exhibited dramatic changes in root hair growth including a decrease in hair length and density. On the other hand, constitutive expression (*CaMV 35S*:*RSL4)* of RSL4 enhanced the root hair elongation and produced long hairs (Yi et al., 2010).

Being a transcriptional regulator, RSL4 could regulate root hair cell growth by controlling the expression of downstream genes that together would form the regulatory network of root hair development. To test this prediction Yi et al (2010) performed a gene-expression study to identify the differentially expressed genes by comparing the transcriptomes of 12 days old wild type, *rsl4* and *CaMV 35S:RSL4* roots using the Affymetrix GeneChip array. 83 genes with significant transcriptional changes upon RSL4 modulation were identified from this preliminary microarray analysis (Yi et al., 2010). All these genes were both down regulated by more than 1.5 fold in the *rsl4* mutants and up regulated by over 1.5 fold in the *35S:RSL4* plants. Further some of the genes (eg: *EXPA 7, PRP3)* identified have been hypothesized to function during cell growth processes that is suggestive of their role in the RSL4 mediated regulation of root hair growth. Hence I utilised this microarray data to perform an independent analysis in order to identify the genes regulated by RSL4 during root hair growth.

5.3 Results

5.3.1 RSL4 regulates the expression of genes involved in cell growth processes

After aligning the 83 putative *RSL4* target genes in descending order (genes arranged based on the sum of the fold change when comparing the gene repression in the *rsl4* mutants with Col-0 and gene activation in the *35S:RSL4* plants when compared to Col-0), I selected a subset of 10 genes that showed the highest sum of fold change, for further characterization (Table 3). In addition to exhibiting a high fold change in relative gene expression when comparing Col-0 vs *rsl4* and *35S:RSL4* vs Col-0, the T-DNA insertion mutants of these genes were readily available.

Of the 10 genes selected, four genes (*RHS4, RHS7, RHS14* and *RHS 18*) have been previously characterised by Won et al (2009) as *ROOT HAIR SPECIFIC* (*RHS*) genes. 19 *RHS* genes were identified. As a result of expression studies and functional analyses a subset of these RHS genes have been suggested to be key players in root hair growth (Won et al., 2009).

Based on these data it can be predicted that RSL4 could regulate root hair growth by regulating the expression of the *RHS* genes. However, the loss of function mutants of the *RHS* genes chosen as part of this study did not exhibit any aberrant root hair phenotype (data not shown). Similarly upon overexpressing the four *RHS* genes except for *35S:RHS18* which developed root hairs that were 20% shorter in length than in Col-0 (Won et al., 2009), the other overexpression lines did not show any change in root hair development. However the phenotype observed for the *35S:RHS18* lines were variable with most of the lines developing wild type root hairs. Hence these results indicate that the selected four *RHS* genes do not play an essential role in root hair development.

Therefore the main focus of this study was to determine the function of the remaining six genes namely: *AT1G13390/EXT12*, *AT1G34510/PRX8*, *AT5G57530/XTH12*, *AT1G26250/EXT18*, *AT4G04900/RIC10* and *AT4G30320* in root hair growth. In order to investigate the role of these genes, I observed the root hair phenotype of the plants that were homozygous for the T-DNA insertions in these 6 genes and in the plants that overexpressed each gene.

Table 3: Potential RSL4 regulated genes selected for function characterization (aligned according to the sum of the fold change).

No	Gene	Fold change Col-0 vs *rsl4*	Fold change *35S: RSL4* vs Col-0	Sum of fold change	Gene product
1.	***AT5G57530/XTH12***	7.9	2.3	10.2	Xyloglucan: xyloglucosyl transferase
2.	***AT1G30850/RHS4***	4.9	2.6	7.5	Unknown
3.	***AT1G54970/RHS7 /PRP1***	3.9	2.4	6.3	Proline rich protein
4.	***AT5G22410/RHS18 / PRX 60***	3.6	2.6	6.2	Class III Peroxidase
5.	***AT4G13390/EXT12***	4.5	1.7	6.2	Proline-rich extensin
6.	***AT1G26250/EXT18***	3.3	2.1	5.4	Proline-rich extensin
7.	***AT4G30320***	3.4	1.9	5.3	V5/Tpx-1-related Allergen
8.	***AT1G34510/PRX8***	2.4	2.7	5.1	Class III Peroxidase
9.	***AT4G04900/RIC10***	3.2	1.9	5.1	Rop-interactive CRIB motif–containing protein
10.	***AT4G22080/RHS14***	2.5	2.2	4.7	Pectate lyase

5.3.2 None of the selected mutants had root hair defects

In order to characterize the biological role of the selected RSL4 regulated genes, I analyzed the root hair phenotypes of the T-DNA insertion mutants by growing them under normal conditions (on the surface of Paul's medium). After confirming the T-DNA insertion of the six genes by genotyping PCR (Primers are listed in Appendix 4), the root hair phenotypes were observed for: *AT1G13390/EXT12* (SAIL_1249_F11), *AT1G34510/PRX8* (SALK_130310), *AT5G57530/XTH12* (SALK_008718), *AT1G26250/EXT 18* (SALK_024549), *AT4G04900/RIC10* (SAIL_564_F11), and *AT4G30320* (SALK_077967). Under normal growth conditions the average root hair length of Col-0 was around 255 µm (mean and standard deviation). Similar to Col-0 the mutant lines of the selected six genes were found to develop root hairs with an average length ranging between 250-260µm. Furthermore the root hairs of these mutants developed from most of the H cells identical to that seen in Col-0 (Figure 5.1, 5.2 and 5.3). These results indicate that the loss of function mutation of the selected putative RSL4 regulated genes did not have any effect on the root hair length or shape.

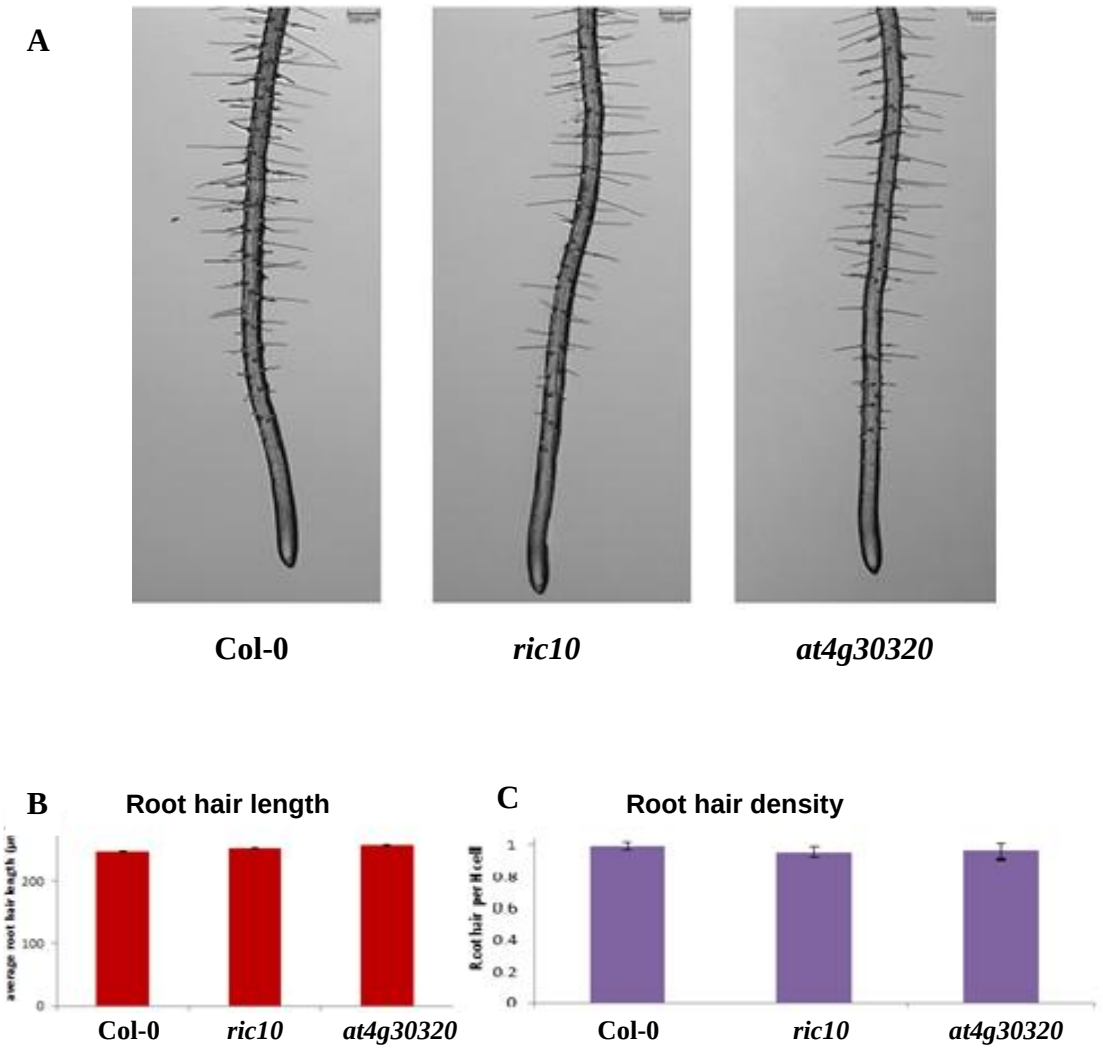


Figure 5.1: *ric10* and *at4g30320* mutants developed root hairs similar to the wild type.

A: Representative bright field images were obtained from 7 day old wild type, *ric10* and *at4g30320* loss of function mutants. **B:** Root hair length of the mutants. Over 1000 root hairs were measured for each genotype, bars represent standard error. **C:** Root hair density of the mutants. For measuring the number of root hairs per H cell file, twenty root hair cells per seedling were scored under the microscope. Ten seedlings per genotype were measured, error bars represent standard deviation. Scale bars, 500µm

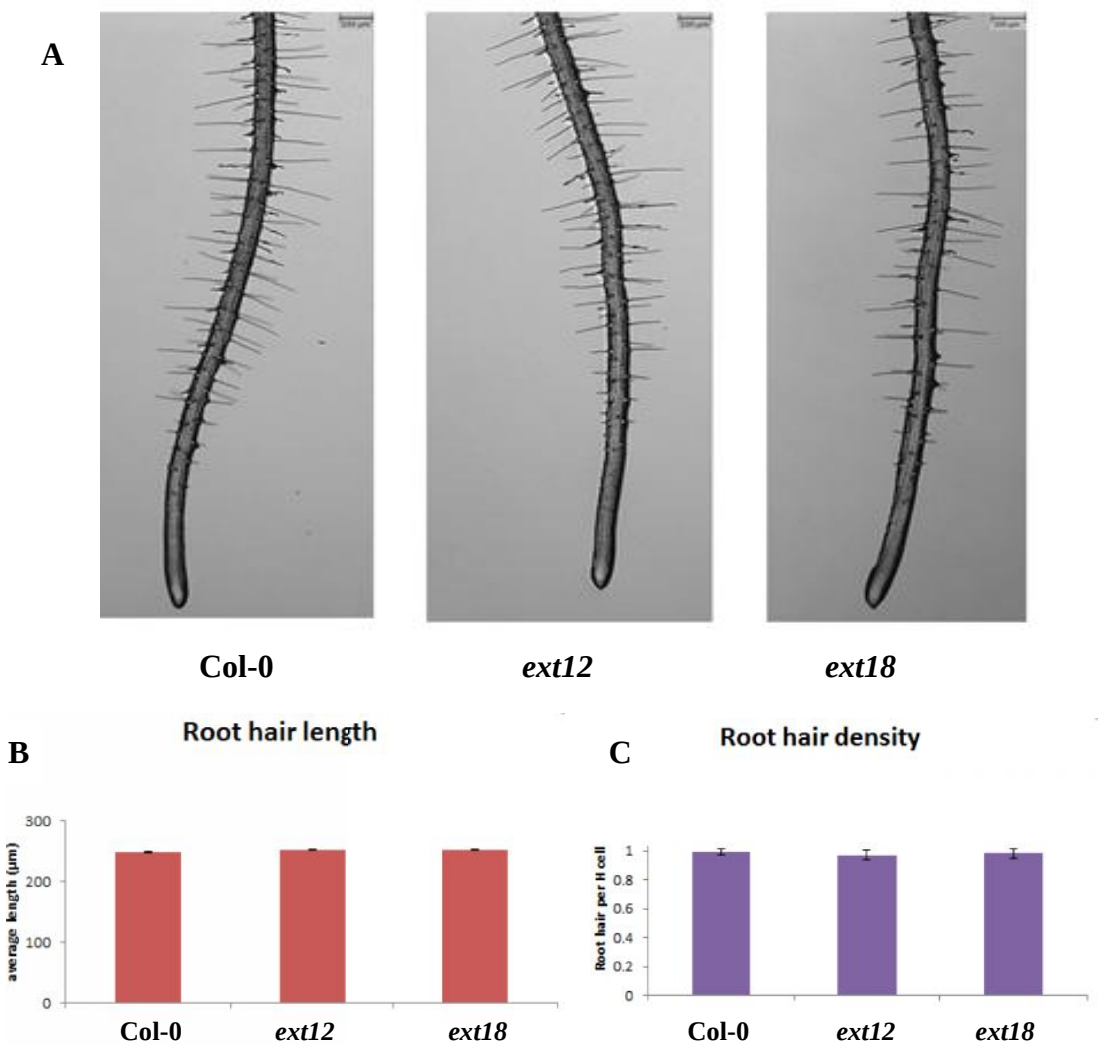


Figure 5.2: No alteration in root hair development was seen in *ext 12* and *ext18*.

A: Representative bright field images were obtained from 7 day old wild type, *ext12* and *ext18* loss of function mutants. **B:** Root hair length of the mutants. Over 1000 root hairs were measured for each genotype, bars represent standard error. **C:** Root hair density of the mutants. For measuring the number of root hairs per H cell file, twenty root hair cells per seedling were scored under the microscope. Ten seedlings per genotype were measured, error bars represent standard deviation. Scale bars, 500µm

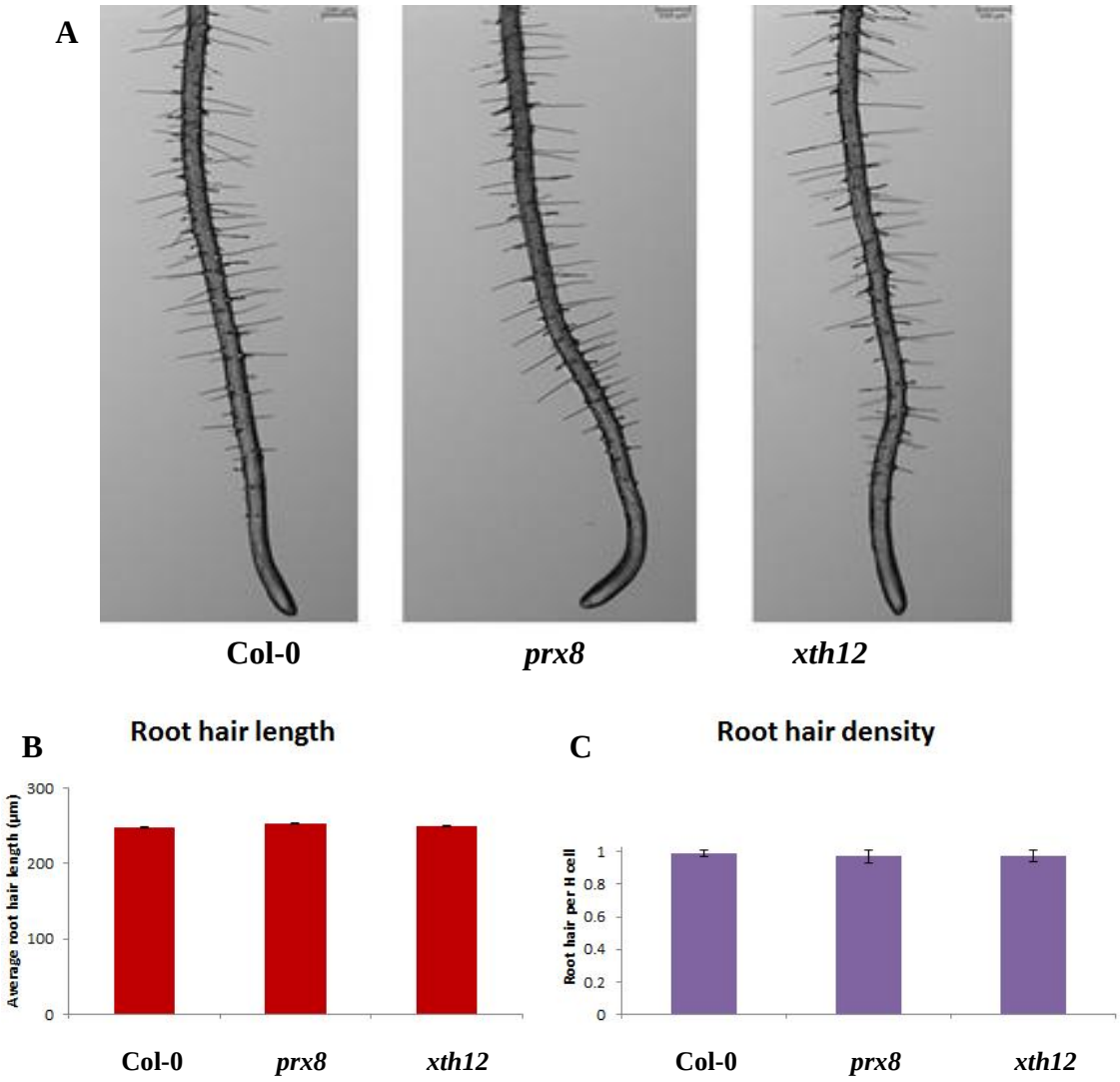


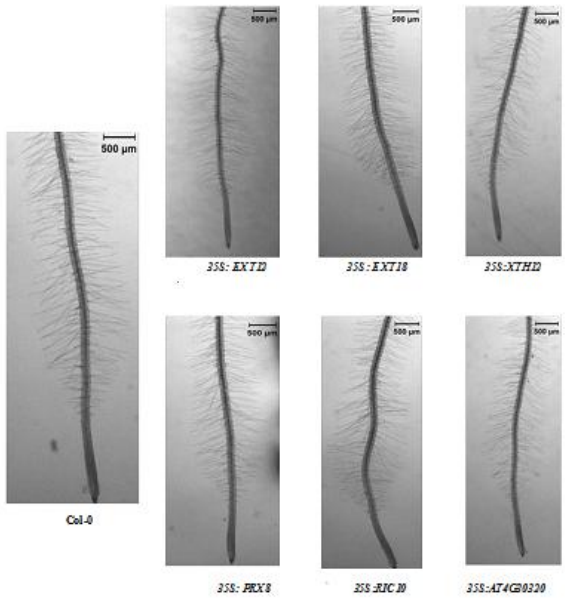
Figure 5.3: *prx8* and *xth12* mutants exhibited normal root hair development.

A: Representative bright field images were obtained from 7 day old wild type, *prx8* and *xth12* loss of function mutants. **B:** Root hair length of the mutants. Over 1000 root hairs were measured for each genotype, bars represent standard error. **C:** Root hair density of the mutants. For measuring the number of root hairs per H cell file, twenty root hair cells per seedling were scored under the microscope. Ten seedlings per genotype were measured, error bars represent standard deviation. Scale bars, 500µm

5.3.3 Overexpression of RSL4 regulated genes do not alter the root hair phenotype

Differential expression studies showed that in addition to being repressed in the *rsl4* mutants the gene expression of these putative RSL4 regulated genes was elevated in the *35S:RSL4* lines. Constitutive expression of *RSL4* results in plants developing longer root hairs when compared to that of wild type plants. This leads to the hypothesis that upon being up regulated *RSL4* could enable constitutive root hair growth by positively regulating the selected six genes. Hence it might be expected that the overexpression lines of the selected genes would exhibit an alteration root hair length, if the genes are regulated by *RSL4* during hair growth. To determine if the gain of function analysis of these six genes could alter the root hair growth, I made constructs to overexpress six genes namely: *AT5G57530/XTH12, AT1G34510/PRX8, AT4G13390/EXT12, AT1G26250/EXT18, AT4G04900/RIC10* and *AT4G30320* driven from the *CaMV 35S* promoter and transferred it into the Col-0 background. After the transformation, I obtained fifteen individual transformants that were selected based on antibiotic screening and PCR. Out of the positive transformants, I selected five individual lines (five seedlings per line) per gene to examine the root hair phenotype. The overexpression lines did not develop any noticeable phenotypic changes in the root hairs (Figure 5.4). This observation was consistent with the root hair phenotype observed with their loss of function mutants suggesting that the chosen genes could function alongside other genes to collectively regulate hair cell growth.

A



B

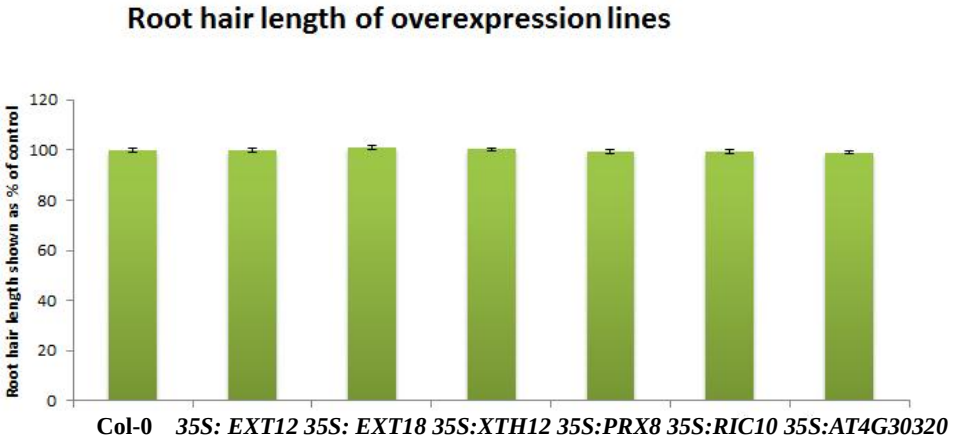


Figure 5.4: Overexpression lines do not exhibit an alteration in root hair development.

A: Represntative bright field images were obtained from 7 day old overexpression lines (Scale bars, 500µm). **B:** Root hair length of the overexpression lines, mature hairs were measured on regions of the root approximately at the same distance from the root tip. The longest hairs in these regions were all of similar length indicating that the hairs had stopped elongating. Twenty five seedlings per gene were measured. Bars show standard error.

5.4 Discussion

Expression studies and phenotypic characterisation have highlighted the essential role of RSL4 as a regulator of root hair cell elongation. However little is known about the mechanism by which RSL4 functions to enable root hair growth. The previous chapter highlighted the identification of the RSL4 regulated target genes using a GR:RSL4 inducible expression system. A parallel approach was taken to obtain a clear picture of what genes are regulated by RSL4 to promote root hair cell growth. A set of 10 genes were selected from the root transcriptomic analysis data that was obtained by comparing the RSL4 loss of function mutant, overexpression line and Col-0 root mRNA. Although the transcriptomic analysis includes all the genes expressed in different cell types in the root, further characterisation by mutant screening coupled with expression studies would filter genes that are specific for hair cell growth. Hence the selected 10 genes were further characterised to understand their specific role during root hair cell development.

5.4.1 Functions of the putative RSL4 regulated genes during root hair cell growth

RSL4 regulates the expression of *AT4G30320* that encodes a pathogenesis-related protein - 1 (PR-1). In *Arabidopsis,* the PR-1 protein family comprises of 22 members that form a major group of the cysteine-rich secretory, antigen 5, and pathogenesis-related 1 protein (CAP) superfamily. These defense-related proteins are induced through the action of signalling compounds such as ethylene or salicylic acid and in response to both the biotic and abiotic stress. As the name suggests one of the main functions of the PR-1 proteins is to elicit defence upon infection. However an increased expression of these PR-1 proteins is seen even in non-infected tissues of healthy plants such as the

root and pollen. Further PR-1 was found to be up regulated in the abscission zone, suggesting that the PR proteins could be involved in cell wall loosening or in the development of the scar tissue against pathogen invasion (Roberts et al., 2000). This indicates that depending on their expression PR-1 proteins may have additional functions in plant cells. Further other PR family proteins such as PR-2, PR-3 in tobacco and tomato seeds play an essential role in the degradation of the cell wall of the seed coat during seed germination along with preventing microbial infection (van Loon et al., 2006). Together these findings serve as evidence to indicate that in addition to defence responses the PR-type proteins could have a role in the plant cell development. To test this hypothesis the role of the RSL4 regulated *AT4G30320* gene in root hair cell development was analysed by observing the root hair phenotype of the corresponding gene mutant and overexpression line. No alteration in root hair cell development upon modulating the *AT4G30320* gene was seen. This indicates that the *AT4G30320* gene has no role in root hair cell growth. Additionally PR genes have been found to possess allergen encoding motifs (Example: Allergen V5/Tpx-1 related, conserved domain) (Ivanciuc et al., 2009) indicating that these PR proteins by cross reactivity with other proteins could also function as allergens that could be responsible for human allergic reaction.

In addition to the PR gene families, cross reactivity among allergens belonging to other protein families such as pectate lyases (PL) and seed storage proteins have been observed (Ivanciuc et al., 2009). This suggests that similar sequence motifs characteristic of allergens are present among the PR genes and the members of the pectate lyase and seed storage gene families. Hence there is likelihood that similar to the PR genes, members of the other genes families could also have a role in plant cell

development. In order to test this hypothesis *RHS 14* encoding a pectate lyase was characterised to understand the gene function in root hair cell development. Pectate lyases are depolymerizing enzymes that catalyse the cleavage of de-esterified pectin which is a major component of the primary plant cell wall (Sun and van Nocker, 2010). Cry j I, a major allergen of Japanese cedar (*Cryptomeria japonica*) pollen has been identified as having pectate lyase activity (Taniguchi et al., 1995). Based on this finding the possible roles suggested for the pollen pectate lyase include the loosening of the pollen cell wall to enable the pollen tube emergence followed by the degradation of the style cell wall to facilitate the penetration of the pollen tube. Similar to pollen tubes, root hairs are an example of cells undergoing tip growth suggesting a role of pectate lyase in root hair cell development.

Further mutations in *PMR6,* pectate lyase-like protein resulted in altered leaf morphology and reduced size of the mutant. This decrease in size is attributed to the defect in the overall cell expansion caused by altered pectin content in the mutant (Vogel et al., 2002). Together these data indicate that PL genes regulate cell expansion by modulating the pectin polysaccharide during the cell wall loosening. However, characterization of the *RHS14* gene function showed no alteration in root hair cell expansion suggesting that *RHS14* does not function in the H cell development.

5.4.2 Functional characterization of cell wall modifying genes indicate gene redundancy

Along with pectate lyase the plant cell wall contains several enzymes that modulate the cell expansion by altering the wall structure (Cosgrove, 2001). Some of these cell wall modifying enzymes include transglycosylases (eg: xyloglucan endo transglycosylase,

XET) that cleave polysaccharides and join them together and peroxidases that could join or break phenolic linkages. *XTH 12* encodes a cell wall enzyme belonging to the *XTH* gene family that function to modify the cell wall by catalysing the cleavage and re-joining of xyloglucan chains (Maris et al., 2010). Based on Promoter::XTH:GUS reporter line analysis, *XTH12* was found to be expressed predominantly in the root hair differentiation zone of the root (Becnel et al., 2006). Additionally purified AtXTH14 and AtXTH26 proteins impaired cell elongation resulting in abnormal root hair morphology when added to growing *Arabidopsis* roots (Maris et al., 2009). Based on these findings it could be predicted that *XTH12* could function in the *RSL4* mediated root hair cell development. However, upon modification *XTH12* did not alter root hair growth indicating that *XTH12* is not involved in the H cell growth. Alternatively *XTH12* could function together with *XTH13* to regulate root hair cell growth as *XTH12* shares a high sequence similarity with *XTH13* (Yokoyama and Nishitani, 2001).

The role of another wall modifying enzyme: peroxidase in H cell growth was demonstrated by Won et al (2009), who showed that on overexpressing *RHS18*, a class III peroxidase the root hair cell elongation decreased by 20% when compared to that of Col-0 (Won et al., 2009). Based on this data they predicted that peroxidases could play an essential role in root hair elongation by mediating the cell wall stiffening. However, I observed that the phenotype of the *35S:RHS18* lines were variable and many lines developed root hairs similar in length to the wild type. Additionally the phenotypic characterization of the T-DNA insertion mutation in another peroxidase gene *AT1G34510/PRX8* selected as part of my analysis showed no change in root hair growth. Peroxidases being a multigene family in *Arabidopsis* several genes could

function together to enable wall modification during root hair cell elongation and hence the absence of a significant root hair phenotype.

One of the reasons behind cell wall stiffening catalysed by peroxidases was attributed to the presence of peroxidase cross linking substrates, like extensins in the cell wall (Won et al., 2009). LEP1, an extensin peroxidase was demonstrated to catalyse high extensin cross - linking emphasizing on the importance of both peroxidases and extensins during cell wall modification (Price et al., 2003; Lamport et al., 2011). Two RSL4 regulated proline rich extensins (plant cell wall structural proteins) namely: *EXT12* and *EXT18* were characterised to determine if they enable cell wall modification during root hair cell growth. No observable changes were seen in root hair growth under the mentioned growth conditions suggesting that these extensins are not too crucial for root hair growth.

Further RSL4 regulated *PRP1* that encodes a proline rich protein and is expressed in root tissues along with PRP3. Histochemical analysis, showed that PRP3 is expressed specifically in differentiating root hair cells and is regulated by developmental pathways leading to root hair growth supporting a role of PRP3 in contributing to the cell wall assembly during hair morphogenesis (Bernhardt and Tierney, 2000). Similarly to determine the role of PRP1 in hair development the root hair phenotypes of the *prp1* loss of function mutant and the *35S:PRP1* overexpression line were observed. The absence of any modification in the development of root hairs in these lines, indicate that PRP1 could function alongside PRP3 in root hair development.

In addition to the modification of the cell wall, tip growth in plant cells such as root hairs and pollen tubes is known to require tip-localized Rho GTPase (Gu et al., 2005). Identification of RICs (Rop-interactive CRIB motif–containing protein), as Rop

GTPase targets helped gain insight into the Rho GTPase dependent signalling pathway occurring during pollen tube growth. Overexpression of *RIC10* greatly promoted the elongation of tip growing pollen tube (Wu et al., 2001) suggesting that it could also have a role in root hair growth. Although the *RIC10* gene expression was found to be regulated by RSL4 no change were observed in root hair development indicating that the RIC10 regulation of cell elongation is restricted to the pollen tubes.

Similar to the other genes, the mutant and the overexpression line of *RHS4* did not exhibit any abnormal root hair phenotype. *RHS4* encodes an unknown protein.

On the whole my results indicate that the selected RSL4 regulated genes are not crucial regulators of root hair cell growth. These genes could encode proteins that could function together with other proteins to control root hair growth or these genes could have no role in root hair development.

CONCLUSION

6. Conclusion

Previous research in my laboratory demonstrated that RSL4 is a key regulator of root hair growth; RSL4 is necessary and sufficient for root hair growth. My initial hypothesis was that RSL4 is part of a regulatory network that ultimately controls the expression of genes that encode proteins with activities required for the growth of the root hair cell (H). According to this hypothesis I predicted that at least some of the targets of the RSL4 regulation would be important for root hair growth, that is the loss of function mutations in these genes would lead to defective root hair growth. In this research project I used two approaches to identify targets of RSL4.

Firstly I introduced an inducible form of RSL4 into mutants lacking the RSL4 function which developed defective root hairs (Chapter 4). Induction of RSL4 activity using dexamethasone (Dex) induced root hair development and the first morphological signs of root hair development were visible within 30 minutes. I have thus developed an inducible form of the RSL4 gene switch that can be exploited for detailed spatial and temporal studies of root hair development. In this thesis I used the inducible form of RSL4 to identify genes whose expression depends on RSL4 activity. Comparison of the transcriptional profiles of roots harbouring the inducible form of RSL4 before and after Dex treatment allowed me to identify genes that are transcriptionally regulated by RSL4. Among these a subset of genes that are likely to be direct targets of RSL4 were identified by using roots treated with Dex and cycloheximide (Cyc) together. Thirty four genes were induced in the presence of both Dex and Cyc and I characterized the phenotypes of plants homozygous for T-DNA insertions into nine of these genes. Four of these mutants developed shorter root hairs than wild type, indicating that their

function is required for root hair development. These include genes that code for proteins involved in cell wall modifications, vesicular trafficking, cytoskeleton organization and signal transduction.

In the second approach I compared the transcriptional profiles from the roots of both the RSL4 gain and RSL4 loss of function plants with wild type to identify genes that were up and down regulated respectively (Chapter 5). None of the genes that I identified using this approach were absolutely required for root hair growth; plants that were homozygous for the loss of function mutations in these putative targets developed wild type root hairs.

On the whole my experimental results demonstrate that my initial hypothesis is correct: RSL4 controls root hair development by coordinating the expression of genes required for growth. My work adds a new tier of genes in the root hair growth regulatory cascade. Characterization of the function of more of these genes will contribute to our understanding of the mechanism of RSL4 regulated cell growth. Furthermore, investigation of the roles of putative orthologs of the RSL4 target genes in other organisms can provide insight into the conservation of RSL4 mediated cell growth processes.

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APPENDIX

8. Appendix

Appendix 1: Primers used to check the GR:RSL4 fusion constructs

Primers within the 5'UTR and promoter of *RSL4*

ATTB4: GGGGACAACTTTGTATAGAAAAGTTGATACGCGTTGGGCTTAAATG
ANTI578: CCTAAAACCTAAACGCGGTG

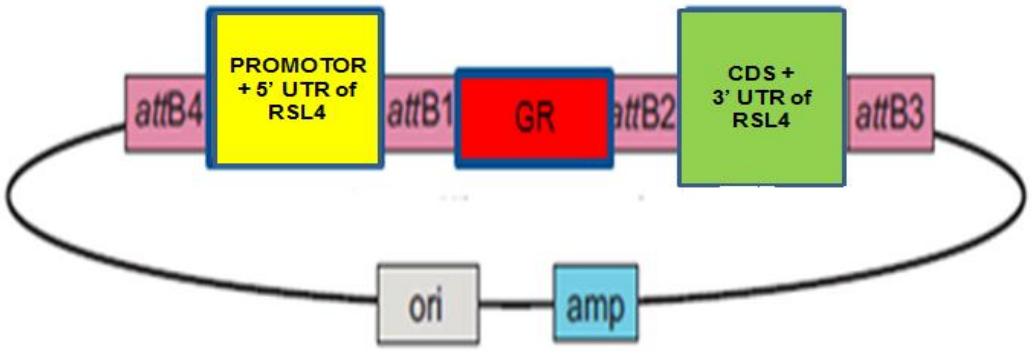
Primers within the CDS + 3'UTR of *RSL4*

RDL2 KpnI: CGGGGTACCATGGACGTTTTTGTTGATGGT
RDL2 BamHI: CTTTTGTCTCGGCTTATGTGAGGATCCGCG

Primers within the GR sequence:

GR forward: CAAAGGGATTCAGCAAGCCAC
GR reverse: CCTTACCTACTGCTTCCAGACATTT

Appendix 2: Schematic diagram of the GR:RSL4 fusion construct used to transform the rsl2 rsl4 mutants in order to generate the glucocorticoid inducible lines



Promoter region: approximately 1kb

Appendix 3: T-DNA genotyping primers for potential RSL4 candidate genes identified from the new microarray analysis (using the GR inducible expression system)

Salk T-DNA line genotyping:

Wild type gene amplification: LP + RP primers were used

T-DNA insertion amplification: LBb1.3 + RP primers were used

LBb1.3-ATT TTG CCG ATT TCG GAAC

No	Gene	T-DNA line	LP primer	RP primer
1.	***AT1G35670/ CPK11/ CDPK2***	SALK_054495 ***(cpk 11-2)***	TTGCAATGTCAC TAATTAACAAAC C	AAACCAATTAGGCG A TGAACC
2.	***AT1G22620/ SAC1***	SALK_083405 **(*sac 1)***	CCC GCA TATACA TCC AAT CTG	GTGAAAGAAGCC AAA AGACCC
3.	***AT5G03540/ EXO70A1***	SALK_014826 *(**exo 70 A1-1)***	TCCACAATAGCT TTGTTCCCT GAA	TGTCACCCACCAAA C CAA ACC
		SALK_135462 **(*exo 70 A1-2)***	CTAGACGTT TGC AGCATCCTAT	ATATGTGTAATGCA T T GGAGAAG C

GABI-KAT T-DNA line genotyping:

Wild type gene amplification: LP + RP2 primers were used

T-DNA insertion amplification: T-DNA + RP1 primers were used

Gene	T-DNA line	LP primer	RP primer	T-DNA primer
AT1G30870/ PRX7	GK_476A09	TCGTAGTAAGGGTGTCCTTCG	**RP1**- GACTACGAAGGGACAGAGAGAAGA	ATATTGACCATCATACTCATT GC
			RP2 -AAGATCTCCGGCTAGCAAAAC	

Appendix 4: T-DNA genotyping primers for potential RSL4 candidate genes identified from previous microarray analysis (Yi et al., 2010)

Salk T-DNA line genotyping:

Wild type gene amplification: LP + RP primers were used

T-DNA insertion amplification: LBb1.3 + RP primers were used

LBb1.3: ATT TTG CCG ATT TCG GAAC

Sail T-DNA line genotyping:

Wild type gene amplification: LP + RP primers were used

T-DNA insertion amplification: LB 3 + RP primers were used

LB3: TAGCATCTGAATTTCATAACCAATCTCGATACAC

No	Gene	T-DNA line	LP primer	RP primer
1.	***AT1G13390/ EXT12***	SAIL_1249_F11	AGTACACACCACACCCAGAGC	AGATGTATGGTGGTGGTGGAG
2.	***AT1G34510/ PRX8***	SALK_130310	TTAAAAATCATCGCTTGCCAG	TGCTCGTTGCTGTATCATCTG
3.	***AT5G57530/ XTH12***	SALK_008718	ATGTCGATGATGGAACTCCAC	ACATCCACGAGTTGCTATTCG
4.	***AT1G26250/ EXT18***	SALK_024549	AAAACTGGTGAGTTGTGGTGC	CGTCGATAGCTACTCACCACC

5.	***AT4G04900/ RIC10***	SAIL_564_F11	TTGAAAGTAAGTGG ATGGTTAATCAC	AGTAGCATCCGGAG AGAGGAG
6.	***AT4G30320***	SALK_077967	AGGTCGGATTTTGC TTAAAGG	AGTTGTGTTTCGGT TGCAATC

Appendix 5: qRT-PCR primers:

No	Gene	Forward primer	Reverse primer
1.	***AT1G34510/ PRX8***	GCATGGGTTTTACGAGGGTA	**R1**- GATGGTCTTTCGGTTGTTGG **R2**- CGACGCTTTTCTCTGATGGT **R3**- TCAAGAGGGAAGCATCACAA
2.	***AT4G30320***	**F1**- CCATTCCAATGGACCCTATG **F2**- CCAATGGACCCTATGGTGAG	**R1**- CGCAACCAATCTTTTGTGTG **R2**- GTGAGCGCAACCAATCTTTT **R3**- GAGCGCAACCAATCTTTTGT
3.	**GAPDH (reference gene)**	CTTGAAGGGTGGTGCCAAGAAGG	CCTGTTGTCGCCAACGAAGTCAG

Appendix 6: Over expression primers

No	Gene	Forward primer (BamH1)	Reverse primer (Sal1)
1.	***AT5G57530 /XTH12***	CGCGGATCCATGGCGG CTTTTGCGACCAA	CGCGTCGACTCAGTTGAGGT TGCATTCAGTG
2.	***AT4G13390 /EXT12***	CGCGGATCCATGATATCC TTAAGAATGAAAGGGC	CGCGTCGACTCAATAATAAG GTGTCTTGTAGATGT
3.	***AT1G26250 /EXT18***	CGCGGATCCATGGCCAGT CCTAATTGG	CGCGTCGACTTAGTATAGTG GTGGAGAGGGAGA
4.	***AT4G04900 /RIC10***	CGCGGATCCATGTCAATG AAAATGAAGGGC	CGCGTCGACCTAGATTAATC GTGATACAGTTGA

No	Gene	Forward primer (KpnI)	Reverse primer (SalI)
1.	***AT1G34510 /PRX8***	CGCGGTACCTGAGGGCA ATCGCAGCTT	CGCGTCGACTCAGTTGTTGA AGGCTCTGCA
2.	***AT4G30320***	CGGGGTACC TGGCTTTT CCTAGTTGTG	CGCGTCGACTCAATAAGGCT TCCTACCAA

Appendix 7: Paul's medium

Pauls's medium, 1L

MS: 4.3g
Sucrose: 10g

Adjust pH to 5.7-5.8 using KOH

Phytagel (0.7%)

Appendix 8: Johnson's medium

Johnson's medium, 1L

Stock A:	10ml
Stock B:	10ml
Inositol (10mg/ml):	10ml
Stock C:	1ml
Stock D:	1ml
MES:	0.533g
Sucrose:	10g

Adjust pH to 5.7-5.8 using KOH

Phytagel（0.5%)

	g/L	**Final concentration**
Stock A（100x）		
KNO_3:	30.3g	3mM
$MgSO_4$:	6.15g	0.5mM
Stock B （100x）		
Ca $(NO3)_2$*$4H_2O$:	47.2g	2mM
Stock C（1000x）		
KCl:	1.864g	25μM
H_3BO_4:	0.773g	10μM
$MnSO_4$*$4H_2O$:	0.223g	1μM
$ZnSO_4$*$7H_2O$:	0.288g	1μM
$CuSO_4$*$5H_2O$:	0.0624g	0.25μM
$(NH4)_6Mo_7O_{24}$*$4H_2O$:	0.309g	0.25μM
Stock D		
$FeSO_4$*$7H_2O$:	6.95g	25μM
Na_2EDTA:	9.365g	25μM

Appendix 9: Potential direct target genes of RSL4 whose relative expression levels are unaffected by Cyc treatment

No	Dex vs Mock	Dex + Cyc vs Mock	Probe ID	Protein/Description
1	2	2.04	***AT1G26940***	Peptidyl-prolyl cis-trans isomerase cyclophilin-type family protein
2	2	2.12	***AT2G25570***	*Arabidopsis thaliana* binding protein
3	2.01	2.23	***AT4G28300***	Hydroxyproline-rich glycoprotein family protein
4	2.02	2.18	***FRS5***	FAR1-related sequence 5; zinc ion binding protein
5	2.03	2.18	***ATSAC1***	Suppressor of actin 1; phosphatidylinositol phosphatase
6	2.12	2.03	***AT4G00830***	RNA recognition motif (RRM)-containing protein
7	2.13	2.08	***AT3G24880***	*Arabidopsis thaliana* DNA binding protein
8	2.15	2.13	***AT1G27360***	Squamosa promoter-binding protein-like 11 (SPL11)

9	2.19	2.24	***AT2G22910***	GCN5-related N-acetyltransferase (GNAT) family protein / amino acid kinase family protein
10	2.2	2.76	***AT1G72880***	*Arabidopsis thaliana* acid phosphatase survival protein SurE, putative
11	2.2	2.42	***AT3G55080***	*Arabidopsis thaliana* SET domain-containing protein
12	2.24	2.71	***ATEXO70A1***	Exocyst subunit EXO70 family protein A1
13	2.25	2.48	***EICBP.B***	Ethylene induced calmodulin binding protein
14	2.25	2.23	***ETFBETA***	ETFBETA, electron carrier
15	2.25	2.36	***PRA1.A2***	PRENYLATED RAB ACCEPTOR 1.A2
16	2.26	2.31	***AT4G26400***	Zinc finger (C3HC4-type RING finger) family protein
17	2.27	2.5	***AT3G13350***	High mobility group (HMG1/2) family protein / ARID/BRIGHT protein

18	2.28	2.35	***AT5G61570***	Protein kinase
19	2.35	2.31	***AT3G18670***	Ankyrin repeat family protein
20	2.38	2.35	***AT4G27620***	Unknown protein
21	2.44	2.39	***AT3G23540***	Unknown protein
22	2.45	2.46	***AT1G21610***	Wound-responsive family protein
23	2.46	2.42	***AT3G17090***	Protein phosphatase 2C family protein
24	2.46	2.48	***AT3G56210***	*Arabidopsis thaliana* binding ,mRNA, complete cds
25	2.59	2.62	***AT1G01800***	Short-chain dehydrogenase/ reductase (SDR) family protein
26	2.61	2.65	***ATCDPK2/ AT1G35670***	Calcium-dependent protein kinase 2

27	2.65	2.74	***VPS35B/AT1G75850***	VPS35 HOMOLOG B
28	2.67	2.65	***AT5G58375***	Unknown protein
29	2.68	2.73	***AT3G57570***	*Arabidopsis thaliana* binding mRNA, complete cds
30	3.01	3.05	***AT3G27700***	RNA recognition motif (RRM)-containing protein
31	3.15	3.19	***AT4G35890***	La domain-containing protein
32	3.84	3.83	***R90101***	Glutathione S-transferase - *Arabidopsis thaliana* (Mouse-ear cress)
33	4.1	4.1	***GBF2/AT4G01120***	bZIP (basic leucine zipper) transcription factor
34	23.96	25.48	***PRX7/AT1G30870***	Cationic peroxidase, putative

Genes highlighted in grey are the subset of 9 genes chosen for the first preliminary functional analysis to determine their role in the RSL4 regulated root hair cell growth.