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BACHELOR THESIS DISSERTATION

«CONTROL OF *ERULUS* AND *PERSEUS* GENES' EXPRESSION IN *ARABIDOPSIS THALIANA*»

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Claire

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LIST OF ABBREVIATIONS

***Table 1**: Abbreviations that used in the text, in the figures and in the tables.*

ABBREVIATION	FULL NAME
ARF	Auxin Response Factor
bHLH	Basic Helix-Loop-Helix
CPC	Caprice (given name to an isolated gene, see details in Introduction chapter)
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
EGL3	Enhancer of GLABRA3
ERU	ERULUS (given name from an isolated gene, see details in introduction)
GFP	Green Fluorescent Protein
GL2	GLABRA2 (given name to an isolated gene, see details in Introduction chapter)
GTP	Guanosine Triphosphate
MES	4-Morpholine Ethane sulfonic Acid
MS	Murashige and Skoog (medium)
NADPH	Nicotinamide Adenine Dinucleotide PHosphate
PCR	Polymerase Chain Reaction
PER	PERSEUS (given name to an isolated gene, see details in Introduction chapter)
PI	Propidium Iodide
PME	Pectin MEthylesterase
RLCK	Receptor-Like Cytoplasmic Kinase
RLK	Receptor-Like Kinase
ROS	Reactive Oxygen Species
RSL4	Root hair defective 6-Like 4 (given name to an isolated gene, see details in Introduction chapter)
RNA	Ribonucleic Acid
t-DNA	transfer DNA
Tris	Tris(hydroxymethyl)aminomethane
TTG1	Transparent Testa Glabra
WER	Werewolf (given name to an isolated gene, see details in Introduction chapter)

ΠΕΡΙΛΗΨΗ

Προηγούμενες μελέτες επιβεβαίωσαν ότι ο παράγοντας μεταγραφής RSL4 εκφράζεται στα ριζικά τριχίδια. Σε αυτή την εργασία επιχειρήθηκε να δοθεί απάντηση στο ερώτημα κατά πόσον το RSL4 μπορεί να είναι υπεύθυνο για την έκφραση πολλών γονιδίων, που είναι ειδικά για τη δημιουργία τριχοβλαστών, όπως είναι τα δύο τύπου υποδοχέα κινάσης γονίδια και συγκεκριμένα τα At5g61350 (με το όνομα *ERULUS*) και At3g07070 (με το όνομα *PERSEUS*) χρησιμοποιώντας αντίστροφη γενετική. Αυτά τα δύο μελετήθηκαν στην παρούσα διατριβή. Αυτές οι δύο κινάσες μελετήθηκαν στην παρούσα εργασία. Η γονιδιακή έκφραση των αμφοτέρων κινασών, εξετάστηκε με αξιολόγηση της δραστικότητας του υποκινητή τους όταν κάθε γονιδιακό προϊόν κατασκευάστηκε σε σύντηξη με GFP που εκφράζεται σε μεταλλαγμένα φυτά *Arabidopsis thaliana* τα οποία δεν εκφράζουν RSL4 (με το όνομα *rsl4-1, knock* out φυτά). Η έκφραση των γονιδίων *ERULUS* και *PERSEUS* ενωμένων με GFP παρατηρήθηκε σαφώς και οδήγησε σε νέο φαινότυπο που αναπτύχθηκε με ριζικά τριχίδια. Έτσι, διεξάχθηκαν διάφορες δοκιμές για την καταγραφή των χαρακτηριστικών του νέου φαινοτύπου μεταλλάκτη, ελέγχοντας το μέγεθος των βλαστών και των siliques των *rsl4-1* φυτών, το φαινότυπο των ριζικών τριχιδίων σε διαφορετικά θρεπτικά υποστρώματα και τελικά συγκρίνοντας σημαντικές διαφορές στην ανάπτυξη των ριζικών τριχιδίων με την τροποποίηση αυτών των θρεπτικών υποστρωμάτων. Η έκφραση των γονιδίων κινασών *ERULUS* και *PERSEUS* παρατηρήθηκε στα φυτά *rsl4-1* που λειτουργούσαν ως knock out (δηλ. δεν παράγουν) στην πρωτεΐνη RSL4. Συμπεράνθηκε ότι η πρωτεΐνη RSL4 δεν είναι ο μόνος μεταγραφικός παράγοντας που ρυθμίζει την έκφραση αυτών των δύο γονιδίων *ERULUS* και *PERSEUS* αλλά φαίνεται να εμπλέκεται και κάτι άλλο.

Στο δεύτερο μέρος των πειραμάτων, φυτά αγρίου τύπου *Arabidopsis* μετασχηματίστηκαν με το promPER::PER-GFP για να εκφράσει την πρωτεϊνη PERSEUS συγχωνευμένη με GFP. Αυτό το προϊόν μετασχηματισμού, παρήγαγε πολλούς ανεξάρτητους απογόνους των οποίον η ταυτοποίηση βασίστηκε σε φαινοτυπικούς ελέγχους και ανάλυση PCR.

Στο τελευταίο μέρος αυτής της διατριβής, επαληθεύτηκε εάν το κατασκεύασμα σύντηξης

promPER::PER-GFP έχει ως αποτέλεσμα δραστική την συνδεδεμένη με GFP πρωτεΐνη PER. Από προηγούμενη εργασία της ομάδας IMPRES του Πανεπιστημίου της Αμβέρσας είχε εισαχθεί το κατασκεύασμα promPER::PER-GFP σε φυτά που είχαν αδρανοποιήσει αυτό το γονίδιο (*Perseus*). Συγκρίνοντας αυτά τα φυτά, με μεταλλαγμένα φυτά *Perseus* που δεν παρήγαγαν την πρωτεΐνη PERSEUS και με φυτά άγριου τύπου, παρατηρήθηκε πως τα φυτά με την εισαγόμενη πρωτεΐνη με σήμανση GFP, είχαν τον ίδιο φαινότυπο ριζικών τριχιδίων με αυτά του άγριου τύπου, οδηγώντας στο συμπέρασμα πως η πρωτεΐνη PERSEUS-GFP μπορεί να αποκαταστήσει το φαινότυπο των ριζικών τριχιδίων σε αυτή των φυτών άγριου τύπου.

ABSTRACT

Previous studies have confirmed that the RSL4 transcription factor is expressed in root hairs. In this work it was attempted to answer the question whether RSL4 may be responsible for the expression of many trichoblasts-specific genes, such as the two receptor-like kinase genes, namely At5g61350 (given the name *ERULUS*) and At3g07070 (given the name *PERSEUS*) using reverse genetics. These two kinases were studied in the present thesis. Expression of both kinase genes was tested by evaluating the activity of their promoter when each gene product was constructed in fusion with a GFP expressed in mutant Arabidopsis plants that did not express *RSL4* (named as *rsl4-1*, knock out plants). Expression of *ERULUS PERSEUS* genes fused with GFP was clearly observed and resulted in new phenotype that grew root hairs. Thus, various tests were carried out to record the characteristics of this new mutant phenotype, by checking the size of shoots and siliques of *rsl4-1* plants, the phenotype of the root hairs on different media, and finally comparing significant differences root hair growth by alterating the constitution of these media. Expression of *ERULUS* and *PERSEUS* kinases genes was observed in the *rsl4-1* plants which were operating as knock outs (i.e. did not produce) to the RSL4 protein. It is concluded that the RSL4 protein is not the only transcription factor that regulates the expression of these two genes *ERULUS* and *PERSEUS*, but it appears that something else must be involved as well.

In the second part of the experiments, wild type Arabidopsis was transformed with the promPER::PER-GFP construct to express the PERSEUS protein fused with GFP. This produced many independent lines of these transformants whose identification was based on phenotyping checks and PCR analysis.

In the last part of this thesis, it was verified whether the promPER::PER-GFP construct results in production of an active GFP-tagged PER-proteins. From previous work from IMPRES group University of Antwerp the promPER::PER-GFP fusion was inserted into plants that had inactivated this gene (*Perseus*). Comparing these plants with *perseus* mutant plants that do not produced PERSEUS protein and with wild type plants, it was observed that *perseus* plants with the introduced GFP-tagged protein had the same root hair phenotype as the wild type, leading to the conclusion that PERSEUS-GFP protein works and can restore wild type root hair phenotype.

1 INTRODUCTION

1.1 The model plant Arabidopsis thaliana

From the large number of plants that have been studied in laboratories, only few can be used as genetic engineering models. The most exploited plant is *Arabidopsis thaliana* and it has several beneficial characteristics that make it a model plant (Figure 1). *Arabidopsis thaliana* is a small yearly plant that is a member of the Brassicaceae family (Meinke et al, 1998). The plant has a short life cycle that yields many seeds, it is rather small in size, contains a limited number of chromosomes, is generally easy to handle and does not require exotic growth conditions. Its height does not exceed 40 cm and the plant can complete its life cycle within 8-10 weeks, depending on the growth conditions (Koutsoubelis, 2016). As a result, 6 generations per year are theoretically possible. The short life cycle makes that offspring is quickly formed and that investigators can see the results of for example genetic experiments fast. The growth of the plant is in the form of rosettes and the available flowers can self-fertilize, producing many seeds (Coen and Meyerowitz, 1991). Each flower can produce 30 - 50 seeds while in the whole plant this number can even reach thousands and their length are 0,5mm when they are mature, produced in slender fruits known as siliques (Meinke et al, 1998). The small size of the plant in combination with its fast growth makes its everyday work easy and fast. In addition, a large number of plants can be treated in controlled conditions due to their small size. The fully sequenced genome and small number of base pairs ($1x10^8$) in the DNA (The Arabidopsis Genome Initiative, 2000) allows investigators to more easily handle the genome as well. Several efficient protocols exist to modify the genome and these mostly make use of *Agrobacterium tumefaciens,* a bacteria that is used to transfer foreign DNA into the Arabidopsis genome (Koutsoubelis, 2016).

1.2 Arabidopsis root anatomy

The anatomy of the primary root is relatively simple in contrast with other plants of the same family. If we observe a root, we will notice three consecutive developmental zones.

First, at the tip there is the root cap (RC) that aids in the penetration of the root into the ground and that serves as sensor of the gravitropic angle of the root. In addition, the cap offers protection for the underlying meristem. The meristematic zone (MeZ) is characterized by a central group of slowly dividing cells, the quiescent center (QC). Around these cells, high proliferation of cells can be seen, which after their proliferation, remain small in size forming distinct cell files. Following the meristematic zone one can distinguish the elongation zone (EZ), (Figure 2). This zone can be split up in the transition zone (TZ), where the cells actively prepare themselves for the next phase, fast elongation where the cells substantially increase their length and volume (Verbelen et al, 2006). Finally, the differentiation zone, also known as the maturation zone, contains cells that gain their final form and can have specific functions (Koutsoubelis, 2016).

Figure 1*: Arabidopsis thaliana.*

A cross-section typically consists of concentric layers of specific cells. From the outer layer to the inner, one can distinguish epidermis, cortex, endodermis, pericycle and vascular

tissue. The epidermis of *Arabidopsis* consists of 16 cells, of which some can create root hairs, as we will discuss below (Dolan et al., 1993). Underneath the epidermis, there are the cells of the cortex, which consist of a single layer of eight cells. In other plants of the same family the cells of the cortex are much more in number (Duckett et al, 1994). The underlying endodermis is a cell line that separates the root from the central cylinder and serves as an apoplastic water barrier due to special cell wall depositions known as the Casparian strip. The cells of the pericycle remain in an embryonic state and are responsible for the production of lateral roots (Lin and Schiefelbein, 2001), (Figure 3). At last, the central cylinder contains cells of the vascular tissue, the xylem and phloem cells, that are responsible for the movement of water and nutrients, respectively (Koutsoubelis, 2016).

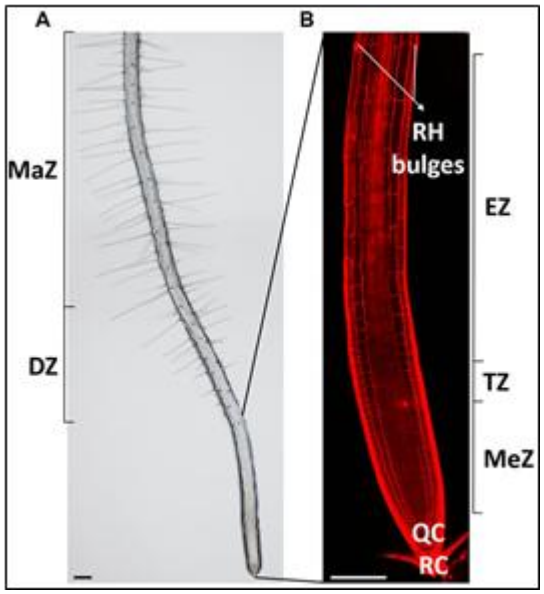

Figure 2*: Overview of the Arabidopsis thaliana root and its developmental zones. (A) Bright-field and (B) confocal picture of a 7-day-old Arabidopsis thaliana root with its distinct developmental zones. RH, root hair MaZ, maturation zone; DZ, differentiation zone; MeZ, meristematic zone; TZ, transition zone; EZ, elongation zone; QC, quiescent center, and RC, root cap. Scale bar = 100 μm (Balcerowicz et al., 2015).*

1.3 Epidermis: trichoblast and atrichoblast cell fates

The *Arabidopsis* root epidermis derives from sixteen initial cells and during maturation these cells are divided into two distinct types of cells: root hair cells (trichoblasts) and non-hair cells (atrichoblasts) (Dolan et al., 1993). Cell fate determination of epidermal cells depends on the location of the epidermis cells in relation to the underlying cortex. The

epidermal cells that are found above the junction between two cortical cells are transformed into trichoblasts (Fig. 3; H) during maturation, while those found above a periclinal cortical cell wall will become atrichoblasts (Fig. 3; N). Since the cortex consists of eight cells, it rarely occurs that two epidermal cells are situated above a cortical cell wall junction so that 2 adjacent trichoblast are seldomly found (Dolan et al., 1993), (Figure 3). The cells that differentiate into trichoblasts are those that create root hairs. These structures help in the absorption of water and nutrients (Clarkson, 1985) by increasing the root's absorptive surface, they provide anchorage for the plant in the soil and provide the sites for symbiotic interactions. From these functions we can understand the importance of the root hairs and how they fulfill important functions for the survival of plants in different environments.

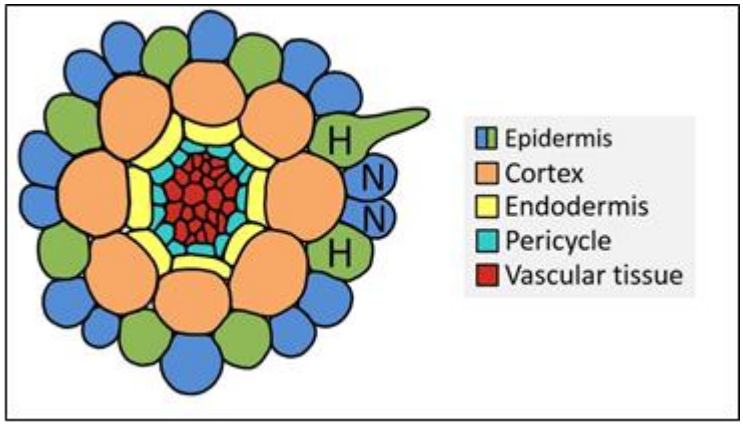

Figure 3*: Cellular organization of the Arabidopsis root. Schematic representation of the cellular organization on a transverse section through the Arabidopsis root depicting the position of root hair (H) and non-root hair (N) cells (Balcerowicz et al., 2015).*

1.4 Root hair cell fate determination

The genetic pathway that regulates root hair cell specification and root hair development is very complex mechanism. There are several components, mostly transcription factors, that are involved in this network. TRANSPARENT TESTA GLABRA (TTG1) forms a complex with the basic Helix-Loop-Helix (bHLH) transcription factor GLABRA3 and ENHANCER OF GLABRA3 (EGL3) (Lin et al, 2015) (Figure 4). This complex will decide the trichoblast or atrichoblast cell fate, depending on its interaction with transcription factors WEREWOLF (WER) or CAPRICE (CPC), which compete for binding to the complex. When WER is bound

to the complex, an N position (trichoblasts) is created, while an H position (trichoblasts) is created when CPC is bound (Song et al, 2011). Typically, WER binding leads to the expression of another transcription factor, known as GLABRA2 (GL2), while CPC interaction inhibits GL2 expression. GL2, in turn, inhibits transcription of the bHLH transcription factor ROOT HAIR DEFECTIVE 6 (RHD6) (Masucci and Schiefelbein, 1994; Menand et al., 2007), which leads to the expression of atrichoblast-specific genes and thus specification of the non-hair fate (N cells). In root hair cells (H cells) RHD6 is expressed so it can promote the expression of RSL4. The protein RSL4 is a basic helix-loop-helix (bHLH) transcription factor that works downstream of RHD6, is sufficient to promote post-mitotic cell growth in root hair cells and its rsl4-1 loss-of-function mutant is hairless (Hwang et al., 2017). RSL4 protein is regulated by hormones like auxin and maybe ethylene and it is a transcription factor that regulates the expression for many trichoblast-specific genes, such as ERULUS and PERSEUS kinases that were discovered in the lab of Professor Vissenberg and which will be discussed later.

1.5 Root hair development

After root hair cell fate is determined, a highly localized bulge is formed that, in later stages, develops into the typical finger-like structure that a root hair is (Figure 5A). Root hair initiation in Arabidopsis begins with the creation of polarity within the epidermal cells and the selection of the initiation site for future root hair outgrowth in the trichoblast cell wall (Carol and Dolan, 2002). Before developing a root hair, at the site where they will develop in the future in the plasma membrane, appear polarly RHO GTPases (RAC / ROP) (Molendijk et al., 2001; Jones et al., 2002). Rising external pH, the bulge initiation process was overturned, indicating that the wall acidification results in the activation of cell wall loosening proteins (Bibikova et al, 1998; Cho and Cosgrove, 2002). Due to the reduced cellulose content, the cell wall loosening and further growth of the root hair is prevented. RHO GTPases are members of one clade, plant RCA / ROPs having a very important role in regulating cell growth and polarity creation, responses for hormones, stress growth, reproduction, and interactions with the environment (Nibau et al., 2006; Yang and Fu, 2007; Kost, 2008). We know important things, such as how active ROP2 can activate ROS-producing NADPH-oxidases, which

controls the evolution of root hairs by altering the architecture of the root cell wall (Foreman et al., 2003; Carol et al., 2005; Duan et al., 2010; Boisson-Dernier et al., 2013) (Figure 5A). By experimentally rising external pH, the bulge initiation process was overruled, indicating that localized wall acidification is required and results in the activation of cell wall loosening proteins.

At the transition between initiation and tip growth (after the bulge formation has been completed), secretion vesicles containing new cell wall material begin to accumulate at the apical part of the bulge, while the highly arranged cytoarchitectural organization at the tip of the growing root hair is built up. The most important part of a hair has a void, which occupies most of the space, while the hemispheric apex is filled with organelles such as mitochondria, Golgi stacks, endoplasmic reticulum and plastids (Figure 5B). A striking tip focused cytoplasmic calcium-gradient is present and is required for root hair tip growth. In the hair, the nucleus follows the growing tip at a constant distance and when the root hair becomes mature, its growth stops and this polar organization of the cytoplasm disappears (Ryan et al., 2001).

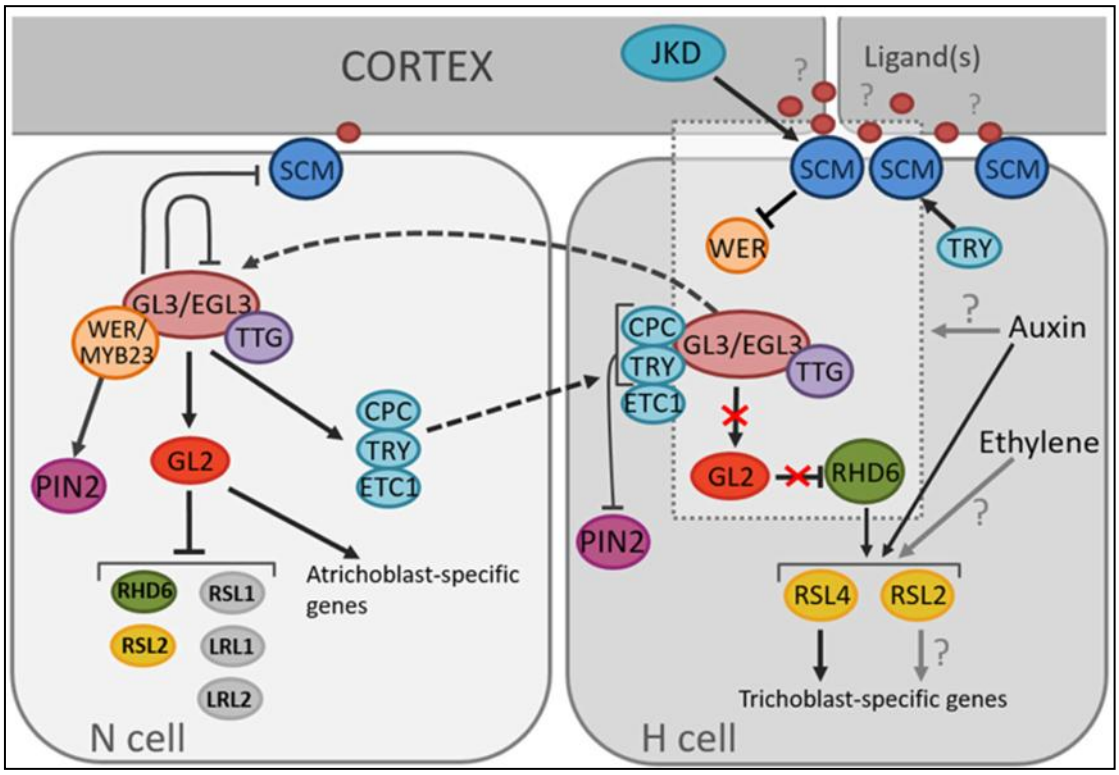

***Figure 4**: Model for the position-dependent cell fate determination of Arabidopsis. Model of molecular pathways determining root hair and non-root hair cell fate in the Arabidopsis epidermis. Arrows, blunted lines and broken lines are indicative of positive control, negative regulation, and intra/intercellular protein movement, respectively (Balcerowicz et al., 2015).*

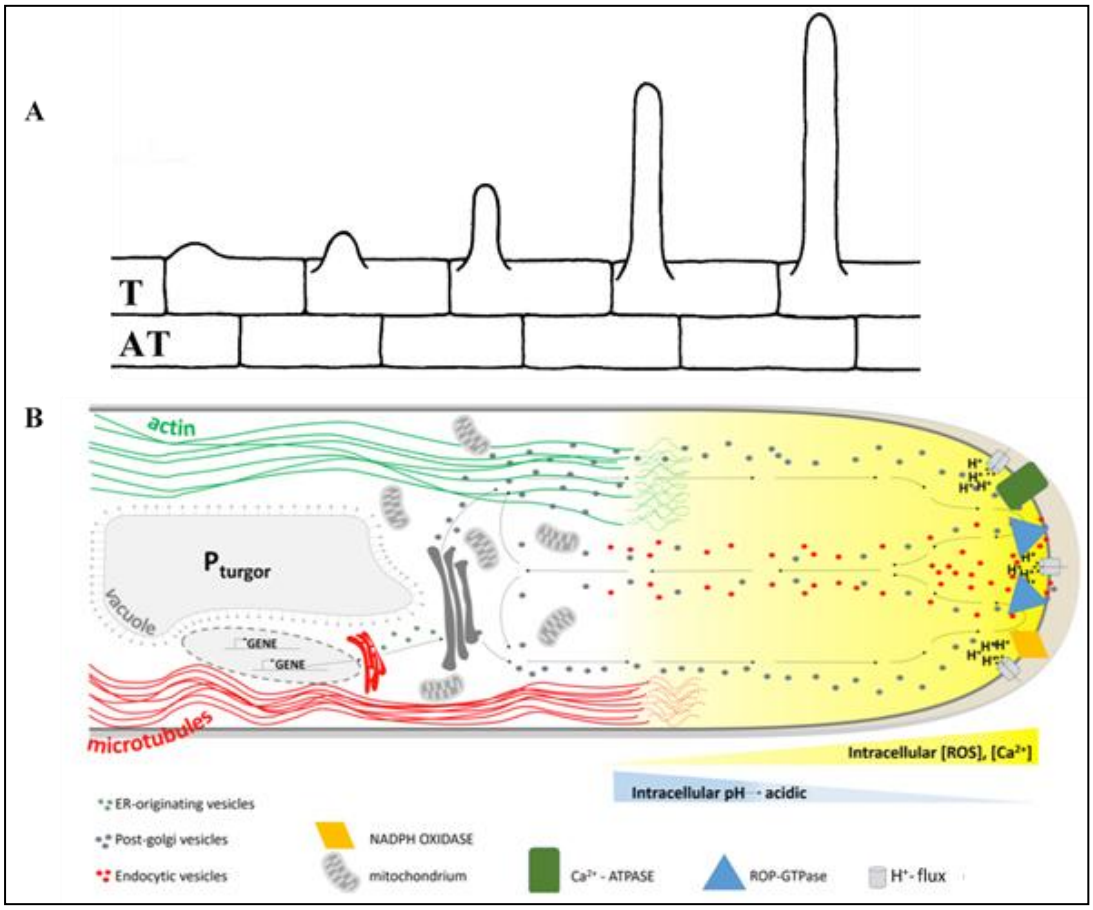

***Figure 5**: Phases of root hair development and scheme of root hair tip growth. **(A)** Different phases of root hair development include the formation of a root hair bulge and subsequent elongation by tip growth. **(B)** This scheme summarizes the events in the cytoplasm at the root hair tip during growth (Balcerowicz et al., 2015).*

1.6 ERULUS and PERSEUS kinases and root hair development

The lab of my promotor has identified and characterized two kinases that play an important role in the development of root hairs.

ERULUS (ERU; At5g61350) belongs to the Catharanthus roseus receptor-like kinase 1-like (CrRLK1L) family that is suggested to contain cell wall integrity sensing receptor-kinases. ERU is exclusively expressed in tip growing cells, namely root hairs (Schoenaers et al., 2018) and pollen (tubes) (Schoenaers et al., 2017) and ERU protein is located in the plasmamembrane of the root hair tip at different stages of growth. The expression of ERU can

be induced by auxin and this happens by direct interaction of auxin-related transcription factors ARF7 and ARF19 with the promoter of ERU. ERU seems to play a role in the fine tuning of specific cell wall enzyme activity, termed pectin methylesterases (PMEs). These change the methylesterification grade of pectin, and hence the presence of Ca2+-binding sites in pectin, which can be visualized by PI staining. In the loss-of-function mutant PME activity was increased and the pectin calcium-binding site oscillations were heavily changed compared to the wild type (Figure 6). Based on the identification of several phospho-targets, this probably occurs by the modulation of proton-pumping activity of H+-ATPases AHA1/2, which change the pH in the cell wall and thus also the activity of pH-dependent PMEs in growing root hair tips. Intriguingly, ERU expression is altered in pectin-related cell wall mutants, suggesting that there exists feedback from the cell wall as well (Schoenaers et al., 2018).

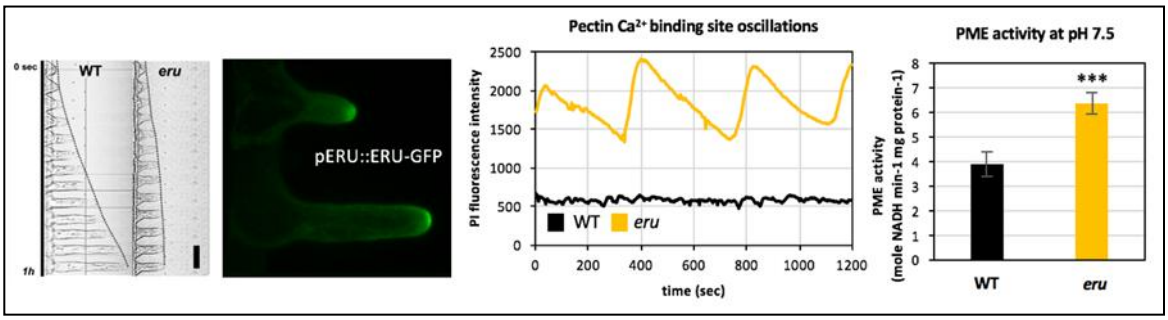

Figure 6*: Summary of ERULUS data. From left to right: Time lapse growth of wild type and eru root hairs, ERU-GFP localisation, demethylesterified pectin oscillations & PME activity in WT (black) and eru (orange) (Schoenaers et al., 2018).*

PERSEUS (PER; At3g07070) was identified in a co-expression analysis for ERU and is assigned to the receptor-like cytoplasmic kinase (RLCK) class VII subfamily, whose members have been reported to play a role in the immune response and in hormone signaling pathways (Swiderski & Innes 2001; Laluk et al., 2011; Lin et al., 2013). *PER* is expressed in the elongation zone epidermis and in root hairs from the beginning of their creation. Like *ERU* its expression is tip growth-specific, but the protein is located in the cytoplasm. Loss-of-function of PER resulted in slower formation of root hair bulges and subsequent small differences in root hair elongation. Experiments where seedlings were grown at different pH

and experiments where proton pumping activity was stimulated or abolished by treatments with fusicoccin or DCCD, respectively, resulted in the hypothesis that PER plays a role in the pH homeostasis of the root hair tips and cell walls. The exact mechanism needs to be further studied. In order to study this, the IMPRES lab built a construct with promoter PERSEUS and protein fusion PERSEUS and GFP (promPER::PER-GFP).

1.7 Objectives

The present thesis focuses on the following axes:

- To find out whether the RSL4 protein is the only required transcription factor that promotes ERULUS and PERSEUS gene expressions.
- To screen for plants expressing the promPER::PER-GFP fusion construct after plant transformation.
- To find out if the GFP-fusion protein of PER coupled to its native promoter (promPER::PER-GFP) can rescue the root hair phenotype in the *perseus* mutant, proving that introduction of this DNA-construct results in a functional yet GFP-tagged PER protein.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

The T-DNA insertion lines in the Arabidopsis thaliana Col-0 background for At3g07070 (PERSEUS; Salk_023374) and At5g61350 (ERULUS; Salk_083442) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The rsl4-1 mutant was kindly donated by Prof. Liam Dolan (University of Oxford, UK). Seeds were first placed in soil for bulking and all experiments were carried out in the growth rooms under controlled conditions (1.5E+15 photons cm-2 s-1, 16 h light/8 h dark, 22°C), (Balcerowicz, 2014).

2.2 Growth media

Plants were grown on full (Table2A) and half-strength (Table2B) solubilized Murashige and Skoog (MS; Murashige and Skoog, 1962) growth medium (Sigma-Aldrich) and on Gilroy growth medium (Table3; Wymer et al., 1997).

Table 2: *Volumes and proportions of 1L medium MS (Murashige and Skoog, 1962).*

A)

COMPONENT	**VOLUME**	**PROPORTION**
M+S (Sigma-Aldrich)	4,3 gr	0,43%
Sucrose	10 gr	1%
MES buffer	0,5 gr	0,05%
Change pH to 5,7 with KOH		
Plant agar	8 gr	0,8%

B)

COMPONENT	**VOLUME**	**PROPORTION**
M+S	2,2 gr	0,22%
sucrose	10 gr	1%
MES buffer	0,25 gr	0,025%
Change pH to 5,7 with KOH		
Plant agar	8gr	0,8%

To make Gilroy, we first prepared individual stocks of the four major inorganics (Table3B), a vitamin stock (Table3C) and a micro-nutrient stock (Table3D). To make Gilroy, the prepared stocks were mixed with sucrose, $(NH_4)_2PO_4$ and after the pH was adapted phytagel was added (Table3A).

***Table 3**: **(A)** Volumes and proportions of 1L Gilroy medium, **(B)** inorganics, **(C)** mixed vitamin stock and **(D)** micro-nutrient stock of Gilroy medium (Wymer et al., 1997).*

(A)

COMPONENT	**VOLUME**	**PROPORTION**
Prepared stock	20 ml	2%
Sucrose	10 gr	1%
1M $(NH_4)2PO_4$	1 ml	0,1%
Change pH to 5.7 with NaOH		
Phytagel	4 gr	0,4%

(B)

COMPONENT	**CONCENTRATION**
KNO_3	1M
$Ca(NO_3)_2$	1M
$MgSO_4.7H_2O$	0,5M
$(NH_4)_2PO_4$	1M

(C)

COMPONENT	**VOLUME**
Thiamine	1 ml
Pyridoxine-HCl	1 ml
Nicotinic acid	1 ml

(D)

COMPONENT	**CONCENTRATION**
KCl	25 µM
H_3BO_3	17.5 µM
$MnSO_4.H_2O$	1 µM
$ZnSO_4.7H_2O$	1 µM
$CuSO_4.5H_2O$	0.25 µM
$(NH_4)6MoO_2.4H_2O$	0.25 µM
Fe-Na EDTA	25 µM
Na_2-EDTA	25 µM

2.3 Seed sterilization

For *in vitro* cultures, seeds were surfaced sterilized in a freshly prepared mixture of 12% bleach and 96% ethanol (1:1 v/v) for 5 minutes. Subsequently, seeds were washed twice in 96% ethanol and allowed to air-dry in the laminar flow.

2.4 Microscopy

A Nikon AZ-100 macroscope equipped with a Nikon DS-Ri1 digital camera was used for all bright field imaging. A Nikon D-Eclipse C1 confocal microscope (Nikon, Brussels, Belgium) was used for fluorescence visualization. Counter staining was done by dipping the seedlings in propidium iodide (0.1 mg ml^{-1}).

2.5 Root hair measurements

Six-day-old seedlings were used for the experiments and ten root hairs per plant were measured with ImageJ software (http://rsbweb.nih.gov/ij/). Statistical analysis was performed using Excel (Microsoft Office PRO Plus 2016).

2.6 Antibiotic selection of transformed plants

During the experiments, screening for homozygous and heterozygous seedlings of T3 generations took place with antibiotics because the homozygous lines were needed for further experiments. For this, ½ MS medium was prepared with 50mg antibiotics per ml medium and the antibiotic for the complementation was Hygromycin (Hyg), while for the protein fusion of construct in wild type plants was Kanamycin (Kan).

2.7 DNA extraction

A number of leaves (1-2) were collected from a seedling in a screw cup tube, frozen in liquid nitrogen and homogenized at 6000 rpm for 10 sec using MagNA Lyser instrument (Roche Life Sciences). After that, samples were centrifuged for 30 sec and 14000 rpm in order to collect the plant material at the bottom of a tube. Next, 150 μl isolation buffer (Table

4) and 100 μl phenol: chloroform: isoamyl alcohol (25:24:1 v/v) were added to each tube what was followed by brief vortexing and centrifugation for 5 minutes at 14000rpm. Supernatant (around 100 μl) was transferred to a clean 1.5 ml Eppendorf tube and DNA was precipitated with two volumes of 100% EtOH. Subsequently, the pellet was collected by centrifugation (12.000rpm, 5 min) and washed with 500μl 70% EtOH. At last, DNA was left to dry overnight in 37^{o}C and rehydrated the next day in 20 μl nuclease-free H_2O.

***Table 4**: Composition of isolation buffer*.

COMPONENT	CONCENTRATION
Tris-HCl (pH 7,5)	50 mM
NaCl	0,3 M
EDTA	20 mM
sarkosyl	2%
SDS	0,5%

2.8 PCR

In order to perform a PCR, the components were placed in a tube (Table 5), doing the necessary actions to not have air bubbles. The PCR machine was regulated (Table 6) and when the temperature was ready, the samples were put inside and the procedure started.

***Table 5**: Final Volumes and final Concentrations of the PCR components.*

COMPONENT	FINAL VOLUME	FINAL CONCENTRATION
5X Green or Colorless GoTaq Reaction Buffer	10 μl	1X (1.5mM $MgCl_2$)$_2$
dNTP Mix, 10mM each	1 μl	0.2mM each dNTP
Upstream primer	x μl	0,1-1 μM
Downstream primer	y μl	0,1-1 μM
GoTaq DNA Polymerase (5u/μl)	0.25 μl	1.25u
Template DNA	Z μl	<0.5μg/50μl
Nuclease -Free water to	50 μl	

The temperature for the annealing step and the time for the extension step were adjusted considering the primers that were used. For the GFP primers, the melting temperature (Tm) was 58.2^{o}C and the product size of these was 395bp.

***Table 6**: Thermal Cycling conditions (Temperature, time and number of cycles).*

STEP	TEMPERATURE	TIME	NUMBER OF CYCLES
Initial Denaturation	95^{o}C	2min	1 cycle
Denaturation	95^{o}C	45sec	25-35 cycles
Annealing	42-65^{o}C	45sec	
Extension	72^{o}C	1min/kb	
Final Extension	72^{o}C	5min	1 cycle
Soak	4^{o}C	Indefinite	1 cycle

2.9 Electrophoresis

Before the electrophoresis procedure can start, a buffer solution had to be prepared, containing a mixture of Tris base Acetic acid and EDTA, known as TAE buffer. To produce this buffer, 100mL 0.5M EDTA solution needed to be produced and for this we used:

- 18.61g EDTA disodium salt (MW = 372.2 g/mol)
- 80mL filtered water
- adjust pH to 8.0 with NaOH
- add filtered water until reaching a final volume of 100ml.

After this, a solution of 50X concentrated TAE buffer was made and for this we used:

- 242g TRIS base (MW=121.14 g/mol)
- 750 ml filtered water
- 57.1ml glacial acetic acid
- 100ml 0.5M EDTA (pH 8.0)
- add filtered water until reaching a final volume of 1l.

When we needed 1L of 1X TAE buffer, 20ml from the 50X TAE was placed in a bottle and sterilized water was put in the bottle until reaching 1L.

Besides TAE buffer, we needed to prepare a 1.5% Agarose gel, by weighing 1.5gr Agarose that was placed in a bottle with 100ml 1X TAE buffer. This solution was placed in the microwave until the agarose was dissolved. The combs were placed in the casting tray and when the agarose was cooled down to about 50-55°C, 6μl/100ml of midori green was added to the agarose solution and this was then poured in the tray before the gel was allowed to solidify (about 20min). To finish, the casting tray was placed in the electrophoresis chamber with the wells closest to the negative electrode. Subsequently, 1X TAE buffer was added to the chamber until the buffer covered the top of the gel and the combs were then removed gently. Carefully, as the length from the DNA that needed to be checked was 395bp, 6 μl of TrackIt 100bp DNA-Ladder (Thermofisher) and then 10μl of each DNA-sample were loaded in the lanes and the power supply was adjusted to 65 volts. The gel started to run and when the samples where reaching the bottom of the gel, the operation was stopped.

3 RESULTS

3.1 Trichoblast-specific expression of RSL4.

As it wanted to make sure that RSL4 was driving the expression of the kinase proteins, the expression pattern of RSL4 checked with the confocal microscope in Arabidopsis wild type plants with the construct promRSL4::RSL4-GFP. Red fluorescence comes from the PI staining to show cell walls (and thus cell boundaries). GFP was clearly present in the nucleus of hair cells only (Figure 7).

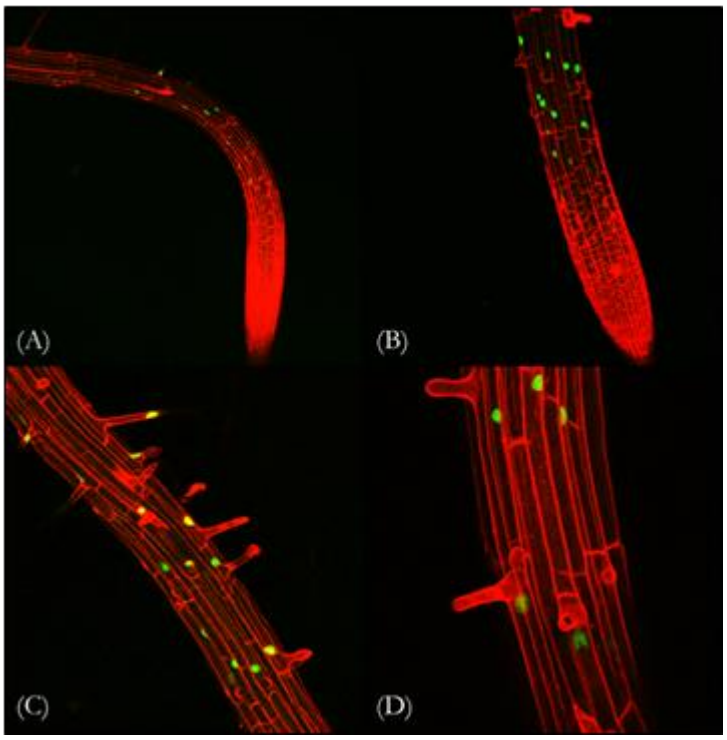

Figure 7: *Appearance of the location of the RSL4-GFP protein fusion in wild type root hair cells using of confocal microscopy depicting the GFP location in cells. The red color is due to the propidium iodide staining, showing the cell wall boundaries. (A): Magnification x10, (B) magnification x20, (C): magnification x40, (D): magnification x60.*

3.2 ERULUS and PERSEUS expression in rsl4-1

Subsequently, because of RSL4's root hair specific expression pattern, my supervisor carried out a cross between *rsl4-1* and promPER::GFP and promERU::GFP to check whether

ERU and *PER* can expressed without RSL4 protein. Before that, it was needed to select those resulting plants that are homozygous for the *rsl4-1* mutation and contain the promPER::GFP construct. The *rsl4*-1 phenotype is characterized by short siliques and a shorter inflorescence stem (Figure 8iA and iiA). As this analysis was already done for *ERU*, it performed the one for *PER*. We therefore grew plants from T3 generation and scored whether they had the *rsl4-1* phenotype. Also, DNA isolated and performed a PCR for the presence of the GFP-sequence with the help of a positive control and GFP-specific primers. After an electrophoresis it could detect which plants had the promPER::GFP construct and it scored the *rsl4-1* phenotype (Figure 9). This analysis identified which lines were homozygous for the *rsl4*-1 mutation and had the desired DNA construct after crossing.

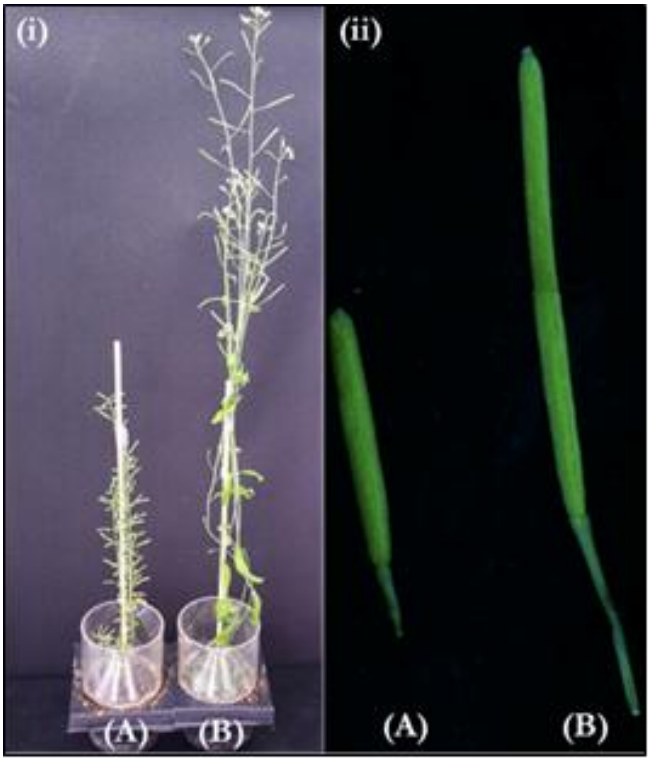

Figure 8: *Arabidopsis thaliana variety(i): Shoots phenotype, (ii) siliques phenotype. (A) rsl4-1 phenotype, (B) wild type phenotype.*

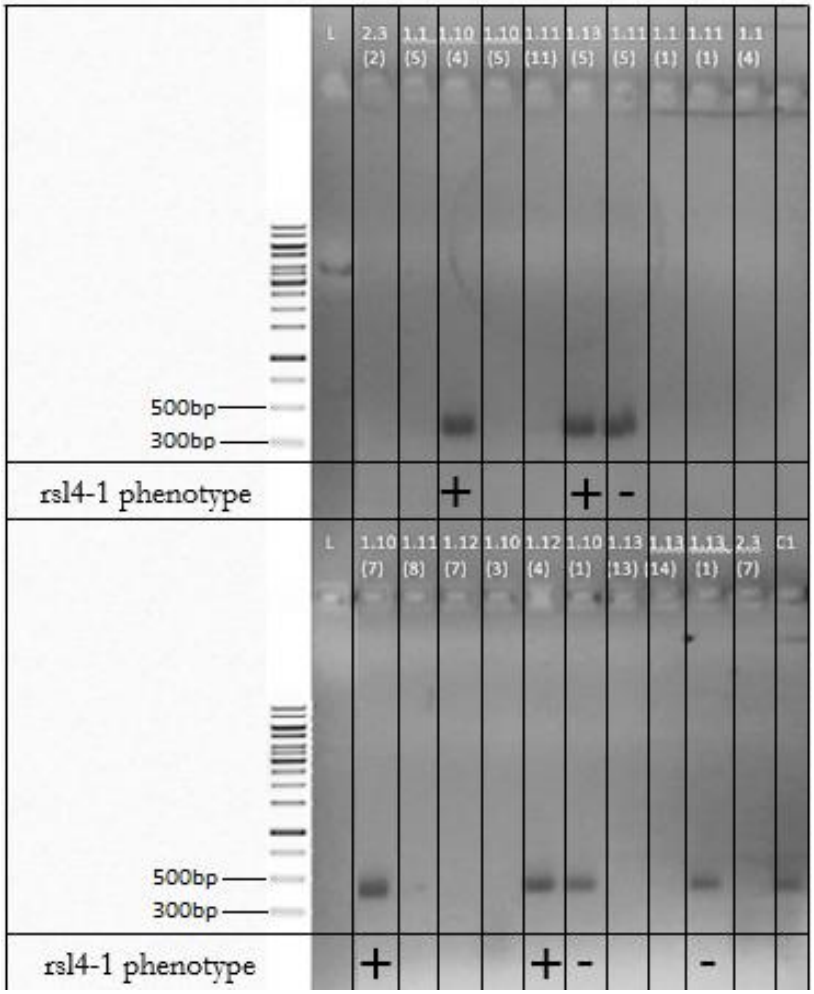

Figure 9: *Electrophoresis gel. Black bands represents the amplified GFP-sequence from the promPER::GFP construct. The coded numbering of the DNA samples represent the following DNA as in the example (e.g.: 2.3(2): 2 represent plant from transformed seeds, 3 represent the seeds after self-pollinated generation, (2) represent the tray's spot of the plant after growing for homozygous searching). +/- represents plants with or without the rsl4-1 phenotype respectively.*

After that analysis, the homozygous lines were selected and they were grown on Gilroy and full MS media with the purpose to verify the expression of promPER::GFP. As the lines for *ERU* were already selected, also they grew to perform the same analysis as for *PER*. Confocal analysis clearly shows that GFP is present for both genes and that the fluorescence intensity was higher when the seedlings were grown on Gilroy than when they were on full MS (Figure 10). To our great surprise, and as can be seen on the images, the resulting plants clearly contain root hairs, that consequently show *ERU* and *PER* expression.

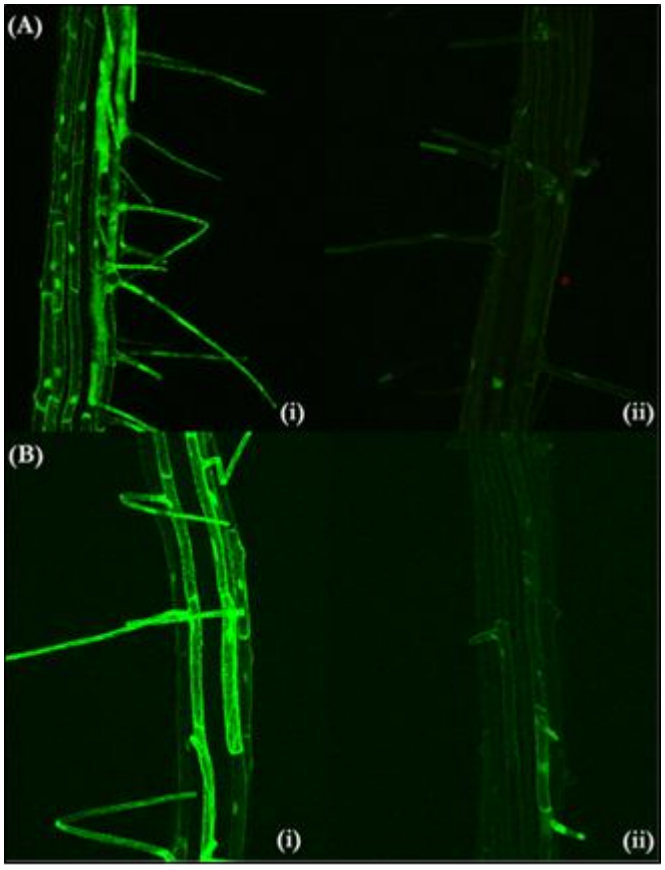

Figure 10: *Photographs from confocal microscopy of: (A) PromPER:GFP cross with rsl4-1 expression in root hairs, (B) PromERU:GFP cross with rsl4-1 expression in root hairs. (i): Gilroy medium, (ii): Full MS medium.*

3.3 Is rsl4-1 working as knock out?

Because root hairs were clearly present on the homozygous *rsl4-1* plants carrying the promoter-constructs, it suggested that maybe *rsl4-1* wasn't really the knock out (so something might have gone wrong during the homozygous selection) and decided to carry out some tests and check if it's true.

The first thing that we checked was the *rsl4-1* phenotype in comparison with wild type phenotype and it was noticed that the crossed plants had clearly shorter shoots and siliques like the right phenotype of *rsl4-1* knock out, indicating that the identified and selected plants were indeed homozygous for the *rsl4-1* mutation (Figure 8).

The second factor that could affect the root hair presence on the roots was the composition of the different growth media. Plants were grown on Gilroy (Fig11), on full MS (Fig12), on half MS (Fig13) while each medium was with and without sucrose added. It was observed on

these different media, there were clearly root hairs present on *rsl4-1*, but that was much less frequent as on the wild type. However, when they had root hairs their length was close to the wild type root hair length, except of full and half MS without sucrose where their growth was not good (Figures 11,12,13).

There were clearly growth differences on the root hairs in these different media and for this reason a comparison of the media was carried out and noticed a big difference in NO_3 and NH_4 concentrations (Table 11). So, NH_4NO_3 and KNO_3 were added to Gilroy to obtain similar concentrations of ammonium and nitrate as in the MS medium. Afterwards, root hair length was verified on control Gilroy and Gilroy with high ammonium and nitrate (Figure 14), which showed that root hair length of wild type and *rsl4-1* decreased when ammonium and nitrate were supplemented.

Also, a Gilroy medium was prepared with a decrease of phosphate and grew plants of *rsl4-1* and *erulus* on this low-P Gilroy and on Gilroy control too. This showed that all lines showed shorter root hairs when grown on low-P medium (Figure 15 and 16).

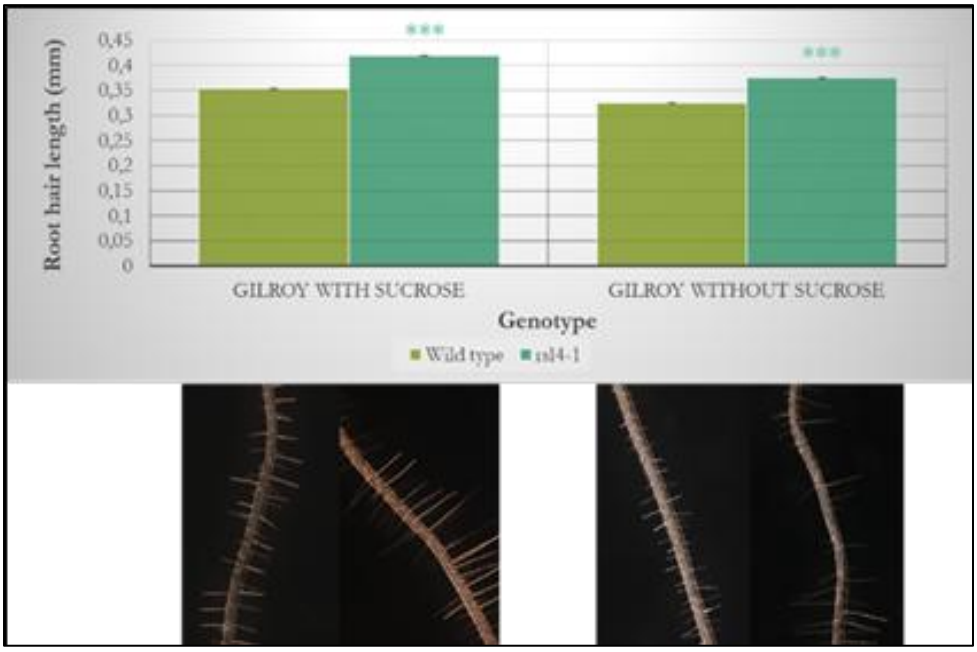

Figure 11: *Root hair length (mm) of different genotypes (wild type, rsl4-1) on Gilroy medium with and without sucrose.*

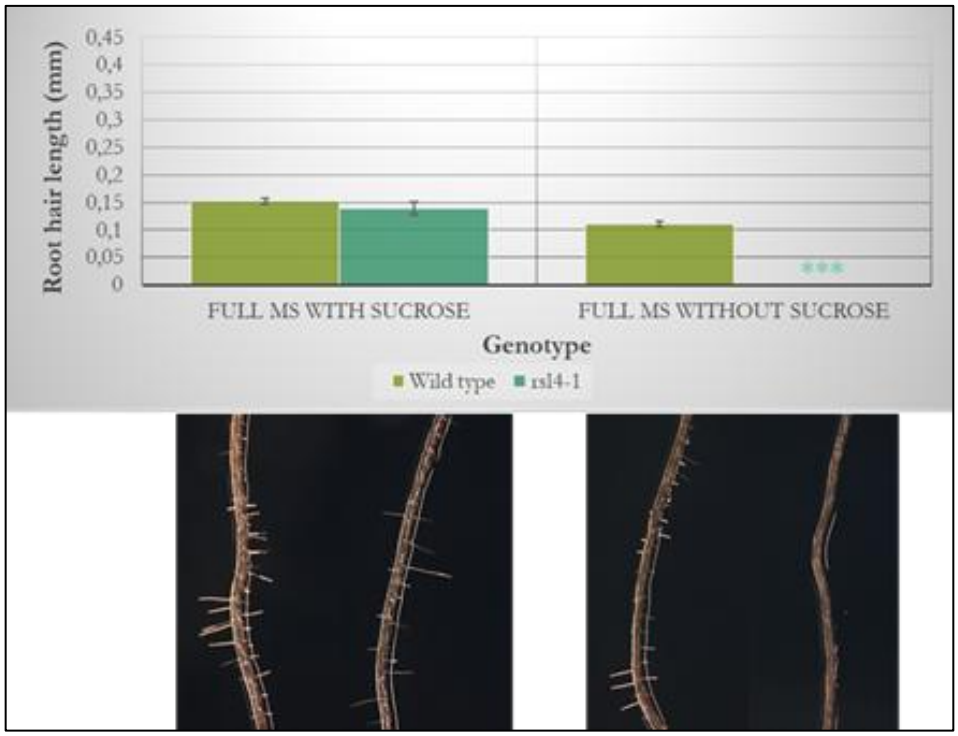

Figure 12: *Root hair length (mm) of different genotypes (wild type, rsl4-1) on full MS medium with and without sucrose.*

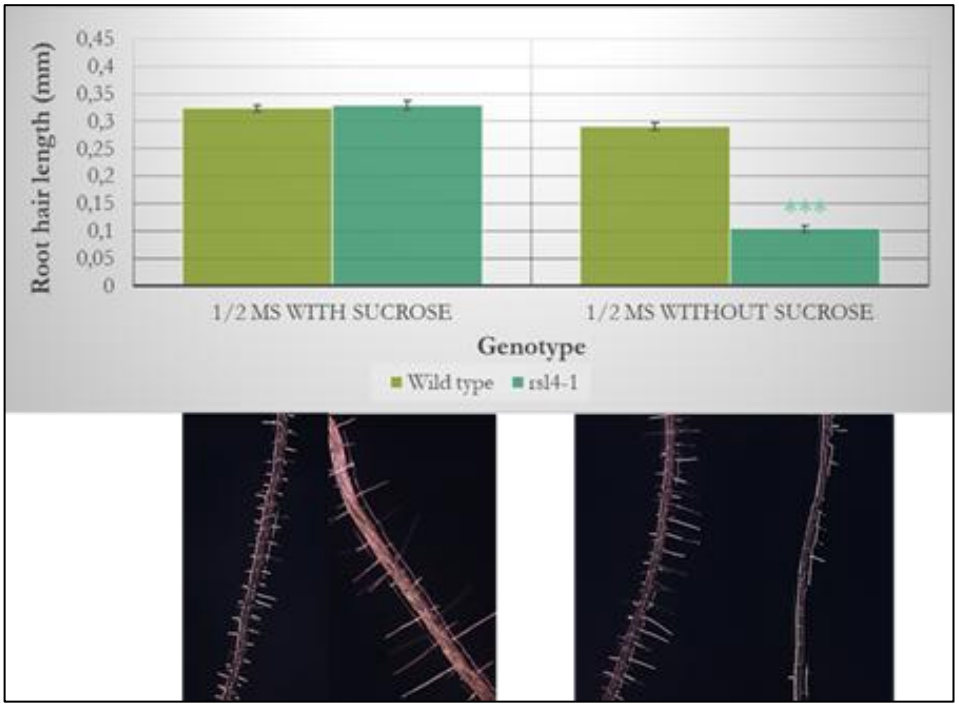

Figure 13: *Root hair length (mm) of different genotypes (wild type, rsl4-1) on half MS medium with and without sucrose.*

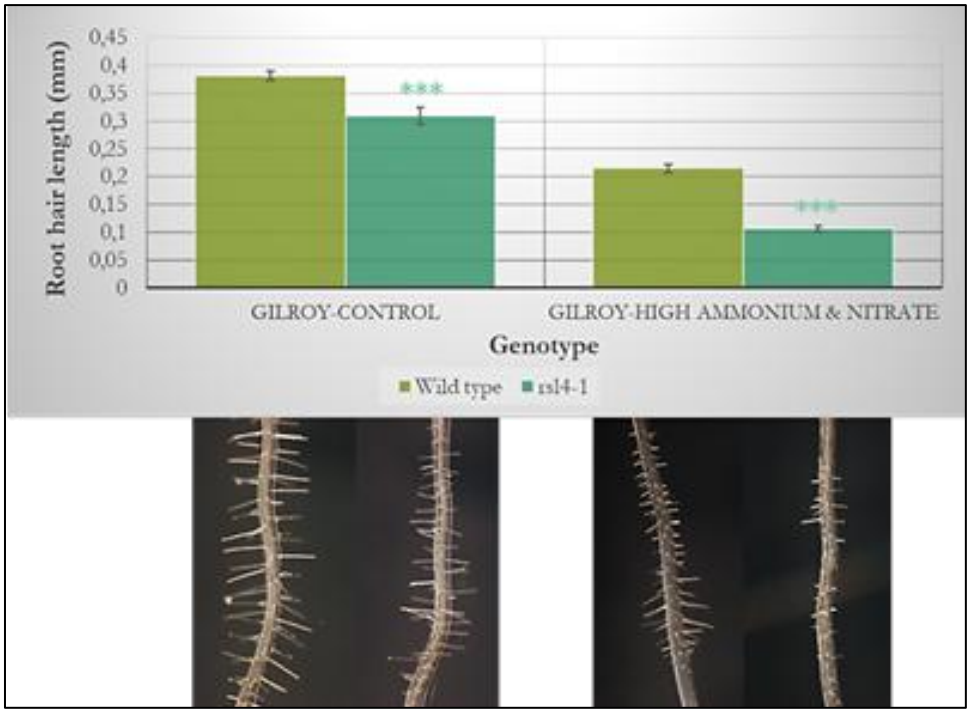

Figure 14: *Root hair length (mm) of different genotypes (wild type, rsl4-1) on Gilroy-control and Gilroy with high ammonium and nitrate.*

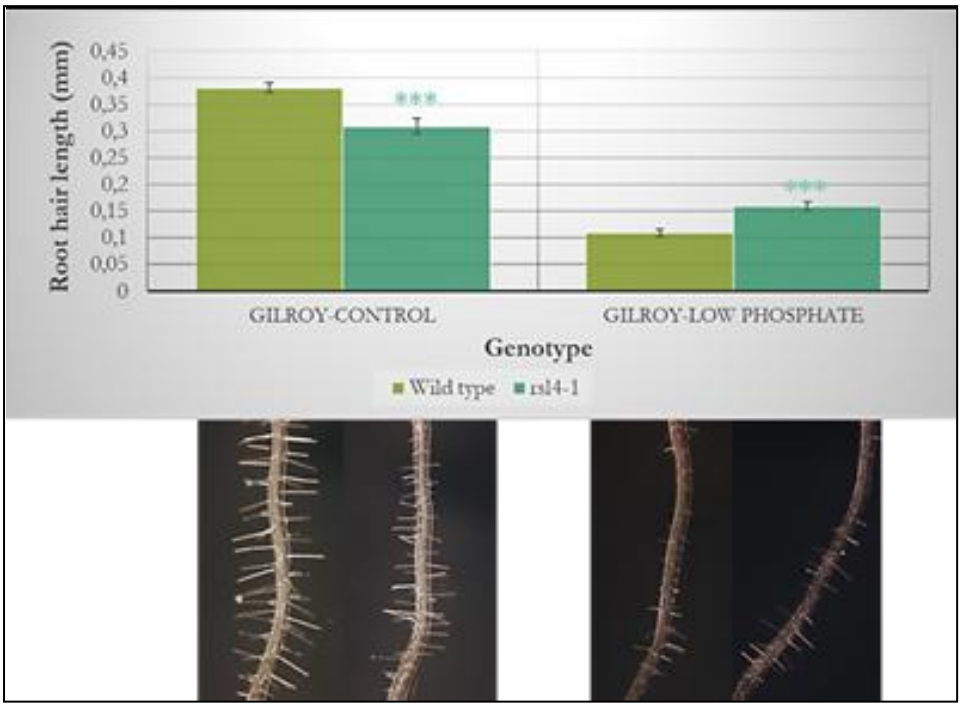

Figure 15: *Root hair length (mm) of different genotypes (wild type, rsl4-1) on Gilroy-control and Gilroy with low phosphate.*

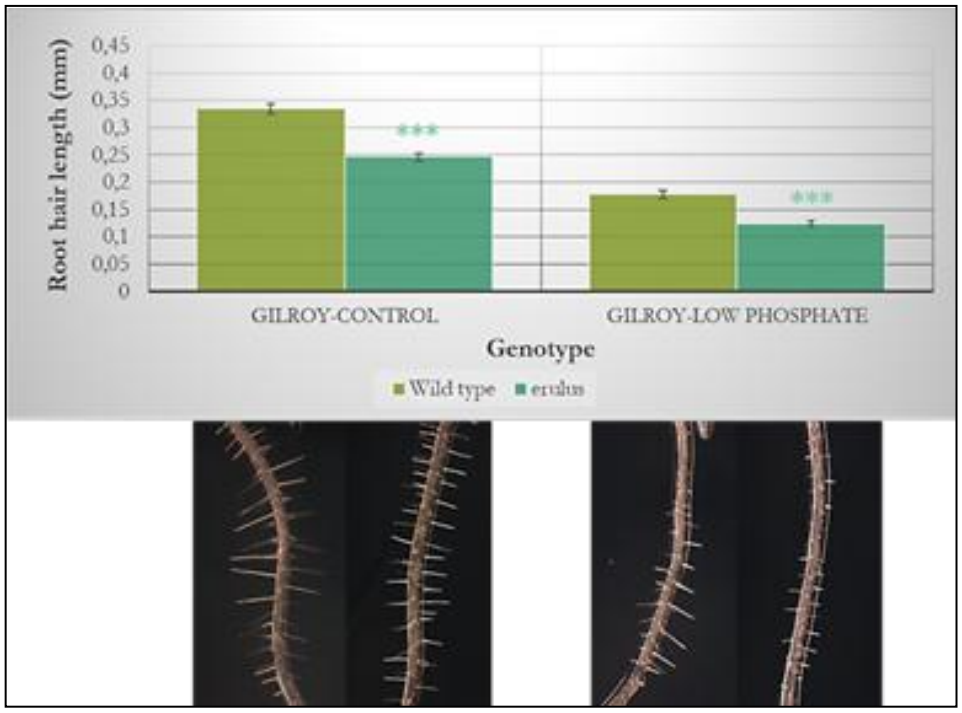

Figure 16: *Root hair length (mm) of different genotypes (wild type, erulus) on Gilroy-control and Gilroy with low phosphate.*

3.4 Screening for homozygous lines after transformation

In parallel with the previous experiments, a screening of T3 generation plants carried out to see which lines were homozygous for promPER::PER-GFP in after transforming the construct into the wild type. So, the lines grew up on half MS medium with antibiotic Kanamycin, since the homozygous lines with T-DNA should have resistance to this antibiotic. The lines that had only alive-green plants were homozygous, these that had alive-green and dead-yellow plants were heterozygous and the lines with only dead plants were wild type. Also, there were some lines with week antibiotic resistance (Figure 17). The results showed that finally they were 10/23 lines homozygous (Table 12).

3.5 Complementation of per by promPER::PER-GFP

Even when the GFP-fusion protein of promPER::PER-GFP is expressed in the wild type, it wasn't known if thus fusion-protein was functional. It could be non-functional because of some reasons, for example if GFP overlaps the active domain of the protein or if the 3D-structure of the protein was changed because of the GFP presence. It was not possible to

check the GFP-fusion protein functionality in wild type because they produce this PER protein on their own. Therefore, it was needed a plant that didn't produce the PER protein. So, the IMPRES group transformed promPER::PER-GFP in the *per* mutant that contains a T-DNA insertion in one of the exons, in an attempt to complement the phenotype (complementation; Figure 18).

Table 11: *Comparison of MS and Gilroy media composition (Murashige and Skoog, 1962; Wymer et al., 1997).*

	MS	**GILROY**
Vitamins [µM]		
Thiamine	0.3	3
Pyridoxine-HCl	2.43	2.43
Nicotinic acid	4.06	4.06
Glycine	26.64	----------
Myo-inositol	554.94	554.94
Macroelements [mM]		
KNO_3	18.79	3
$Ca(NO_3)^2$	----------	2
MgSO4	1.5	0.5
$NH_4H_2PO_4$	----------	1
NH_4NO_3	20.61	----------
KH_2PO_4	1.25	----------
$CaCl_2$	2.99	----------
NO_3^-	39.4	7
SO_4^{2-}	1.5	0.5
PO_4^{3-}	1.25	1
NH_4^+	20.61	1
Microelements [µM]		
H_3BO_3	100.27	17.5
CuSO4.5H2O	0.1	0.25
ZnSO4.7H2O	29.91	1
MnSO4.H2O	100	1
FeNaEDTA	100	25
KCl	----------	25
KI	5	----------
Na2MoO4.2H2O	1.03	----------
(NH4)6MoO24.4H2O	----------	0.25
CoCl2.6H2O	0.11	----------
Na2-EDTA	----------	25

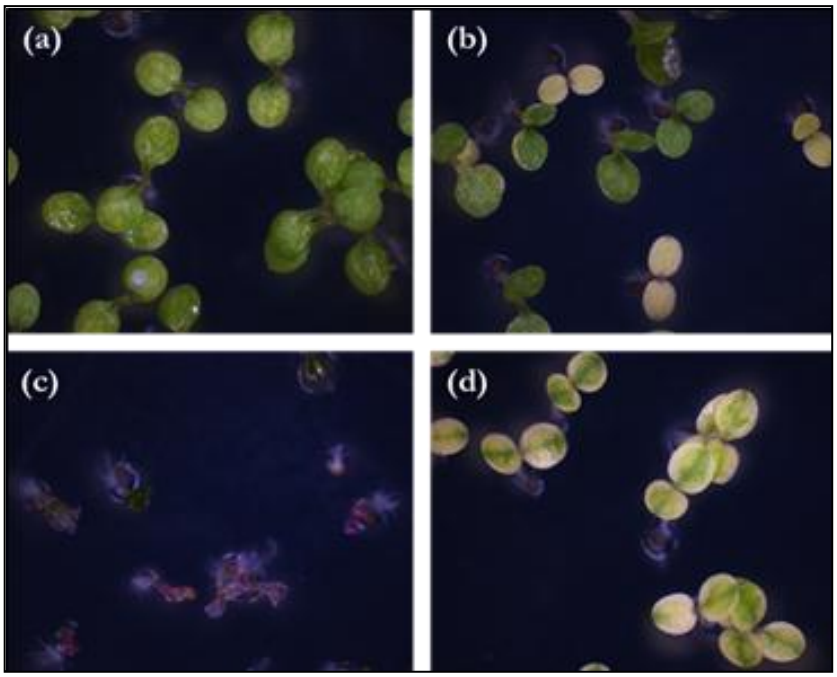

Figure17: *Screening plants of the transformation of wild type with promPER::PER-GFP construct observing the first stage leaves. (a) Homozygous, (b) heterozygous, (c) wild type, (d) weak antibiotic resistance.*

Table 12: *Different lines of plants (1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 3.1, 3.2, 3.3, 3.4, 4.1, 5.1, 5.2, 5.3, 5.4, 5.5, 8.1, 8.2, 8.3, 8.4, 8.5) with their genotype after screening on half MS medium with the antibiotic Kanamycin.*

LINES	**GENOTYPE**
1.1	Heterozygous + Weak antibiotic resistance
1.2	Weak antibiotic resistance
1.3	Heterozygous + Weak antibiotic resistance
1.4	Weak antibiotic resistance
1.5	Weak antibiotic resistance
1.6	Heterozygous + Weak antibiotic resistance
3.1	Heterozygous
3.2	Heterozygous
3.3	Heterozygous
3.4	Homozygous

4.1	Homozygous
5.1	Homozygous
5.2	Homozygous
5.3	Homozygous
5.4	Homozygous
5.5	Homozygous
8.1	Weak antibiotic resistance
8.2	Weak antibiotic resistance
8.3	Weak antibiotic resistance
8.4	Weak antibiotic resistance
8.5	Weak antibiotic resistance

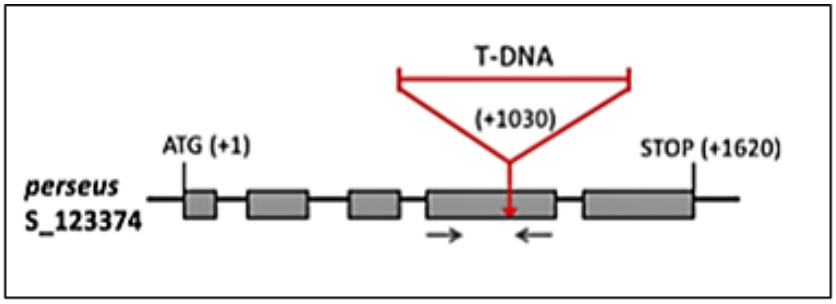

Figure 18: *Characterization of perseus mutant (Balcerowicz, 2014).*

First, a screening of these plants was carried out, to find the homozygous lines with promPER::PER-GFP in *per*. So, the lines grew up on half MS with the antibiotic Hygromycin (Hyg), they were placed in the light for 6 hours and then in the dark. After five days, the plants that were alive had long hypocotyls because of the light incentive that it pushed them to find the light. So, the lines that had only long hypocotyls were homozygous and these that had also short hypocotyls were heterozygous (Figure 19). The results of the screening is that I identified 13/27 homozygous lines (Table 13).

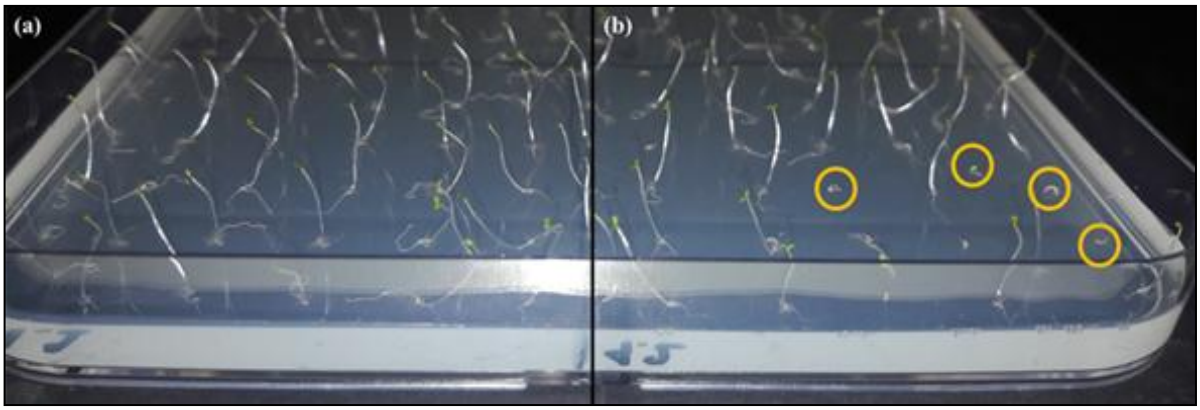

***Figure 19**: Screening plants of the cross with promPER::PER-GFP with per. (a):Homozygous, (b): heterozygous. Plants not resistant to antibiotics are marked with the oranges circles.*

***Table 13**: Different lines of plant 4 (4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.10, 4.12, 4.14, 4.15, 4.16, 4.17, 4.18, 4.21, 4.22, 4.23, 4.24, 4.25, 4.26, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32) with their genotype after screening on half MS medium with the antibiotic Hyg.*

LINES	GENOTYPE
4.1	Homozygous
4.2	Heterozygous
4.3	Heterozygous
4.4	Homozygous
4.5	Heterozygous
4.6	Homozygous
4.7	Heterozygous
4.8	Homozygous
4.10	Heterozygous
4.12	Homozygous
4.14	Homozygous
4.15	Heterozygous
4.16	Heterozygous
4.17	Heterozygous
4.18	Heterozygous
4.21	Heterozygous
4.22	Heterozygous
4.23	Heterozygous
4.24	Homozygous

4.25	Homozygous
4.26	Heterozygous
4.27	Homozygous
4.28	Heterozygous
4.29	Heterozygous
4.30	Heterozygous
4.31	Heterozygous
4.32	Homozygous

After screening, homozygous lines were selected and 3 of them were grown up on Gilroy medium together with *per* and wild type seeds to compare root hairs that were formed. When measurements were done, it noticed that complementation lines had root hairs that were looking like wild type (Figures 20,21 and 22).

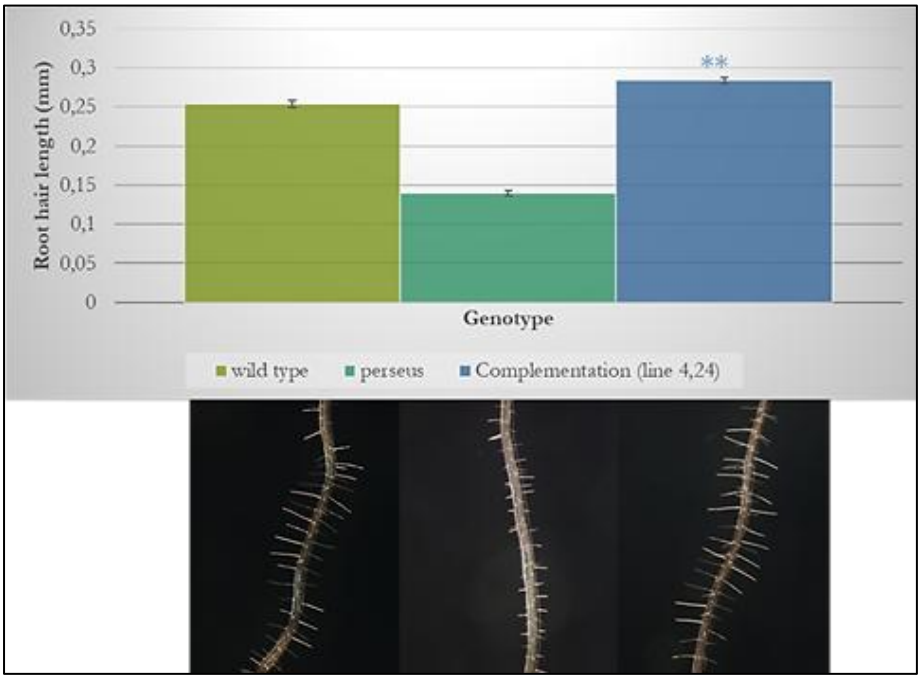

Figure 20: *Root hair length (mm) of different genotypes (wild type, per and complementation line 4.24) on Gilroy medium.*

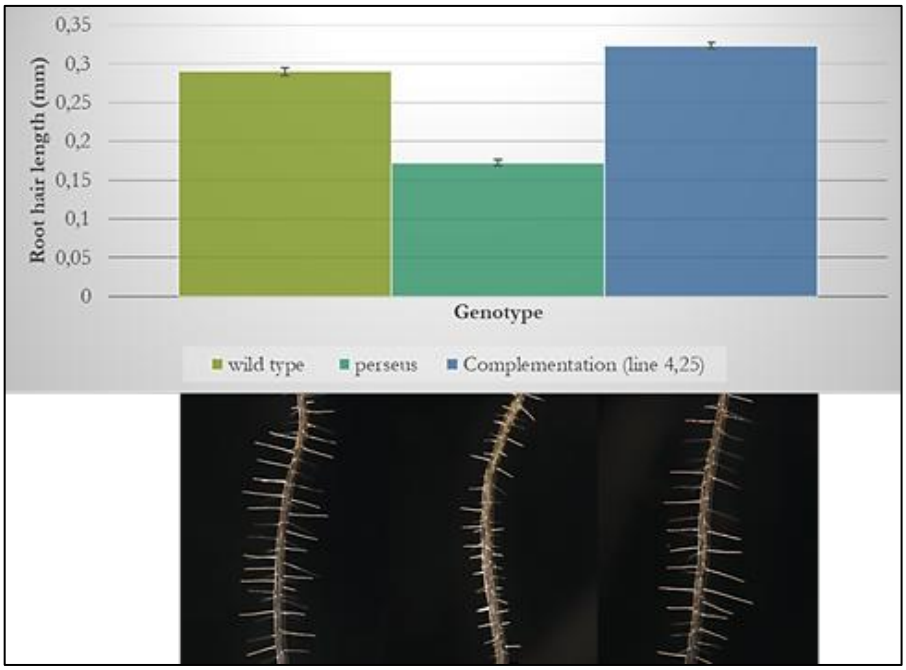

Figure 21: *Root hair length (mm) of different genotypes (wild type, per and complementation line 4.25) on Gilroy medium.*

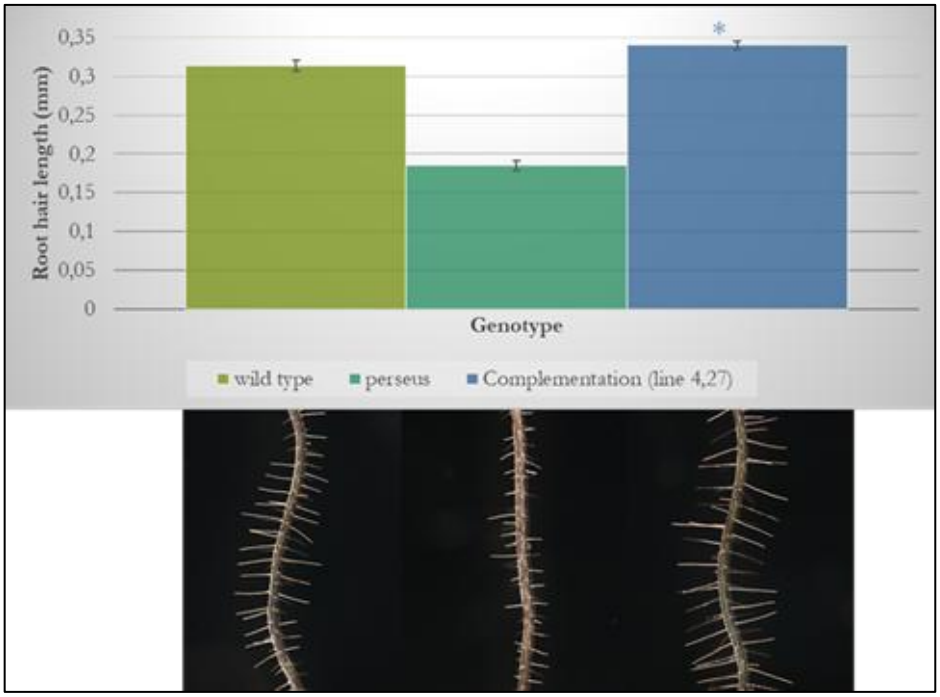

Figure 22: *Root hair length (mm) of different genotypes (wild type, per and complementation line 4.27) on Gilroy medium.*

To gain insight into the function of the PER protein and the possible effect of the receptor

kinase on root hair growth, detailed pictures of root hairs were taken and we noticed that the root hairs of three complementation lines had the same length and morphology as the wild type (Figure 23 a, c), while the *per* mutant, used as a negative control, had shorter root hairs that occasionally branched (Figure 23b).

Growing these lines on Gilroy medium and using the confocal microscope tried to detect GFP in them, but since the expression was so low, it could not reliable visualize the GFP. To do so, it need a more sensitive microscope like the spinning disc that was used for the detection of ERU-GFP in Schoenaers et al. (2018).

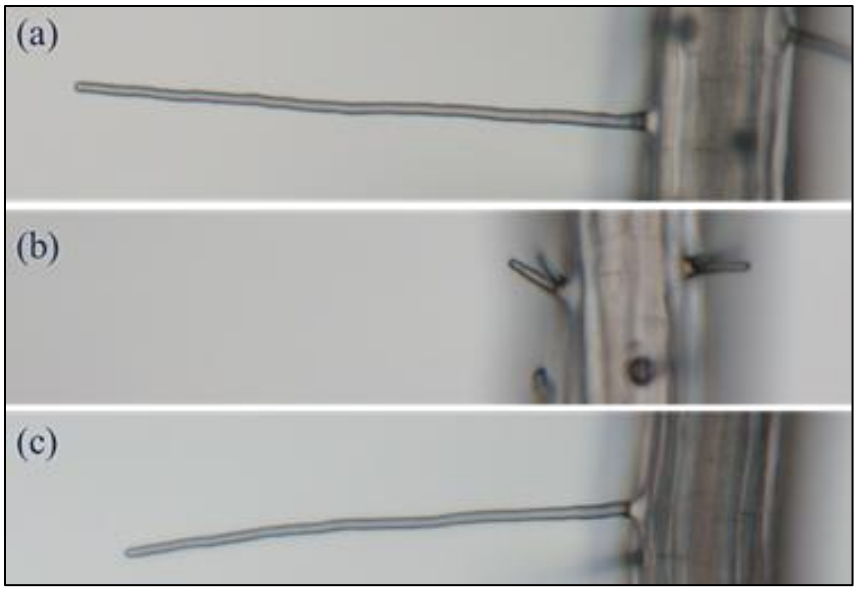

Figure 23: *Microscope images of root hair morphology of a) wild type, b) perseus and c) complementation.*

4 DISCUSSION

"The important thing is to never stop questioning"

Albert Einstein

From previous experiments by other research groups it was known that ERU and PER are working downstream of RSL4 and that when RSL4 is produced, then these trichoblast-specific genes are expressed and root hairs are formed. Therefore, whether these genes were expressed in the *rsl4-1* knock out mutant checked. After checking the expression of promPER::GFP and promERU::GFP in *rsl4-1*, noticed that the promoters of ERU and PER can be induced without this RSL4 protein. First, it thought that our identified crossed lines were not really homozygous for the *rsl4-1* knock out status. From the tests that we carried out, it concluded that the resulting crosses were homozygous for *rsl4-1* since the phenotype of the plants seemed to had short shoots and siliques. Secondly, it could be that something in the medium was inducing *ERU* and *PER* genes. From the test that were done with the different concentrations of certain compounds of the media, it noticed that the expression level of these genes depends on the concentrations of the medium since the plants had different root hair lengths. Therefore *rsl4-1* plants grew on different media and it was root hair formation on the knock out plants that was unexpected, since *rsl4-1* was described to be root hairless. After these results, came to the conclusion that RSL4 is NOT the only transcription factor required for root hair formation and promoter activity of ERU and PER, which was the first aim of this thesis. There must be another transcription factor that in some cases, dependent on the composition of the medium, takes over the role of RSL4. RSL2 could be such a candidate. Therefore, it need to cross the promERU/PER::GFP lines into a *rsl4-1rsl2* double mutant.

T3 generation plants screened successfully after transformation of promPER::PER-GFP in wild type and finally identified 10/23 homozygous lines, which was the second aim of this thesis. These will be used for future localization studies of PER, but a sensitive microscope is needed since the expression level is very low. As a result, the confocal laser immediately kills any possible fluorescence so that cannot see the GFP localization.

Subsequently, the screening of promPER::PER-GFP in the *per* mutant identified 13/27 homozygous lines, which means that the complementation transformation was successful too.

In addition, after comparing root hair lengths between wild type, *perseus* and complementation, noticed that root hairs of 3 chosen complementation lines were looking like wild type, so the tagged PER protein was working well and able to overcome the *per*-mutation.

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