Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Characterization of bHLH transcription factors that control tissue differentiation in *Physcomitrella patens*

Bruno Miguel Grou Catarino

Dissertação de Mestrado em Biologia Celular e Biotecnologia

2012

Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Characterization of bHLH transcription factors that control tissue differentiation in *Physcomitrella patens*

Bruno Miguel Grou Catarino

Dissertation supervised by:

Professor Liam Dolan (Department of Plant Sciences, University of Oxford)

Professor Rui Malhó (Departamento de Biologia Vegetal, FCUL)

Dissertação de Mestrado em Biologia Celular e Biotecnologia

2012

Aknowledgments

First of all, thank you Liam for allowing me to work in such a great lab, with excellent people and for providing me all I needed to develop my projects. You have inspired and guided me during this year with all your knowledge.

To my co-supervisor, Rui Malhó, thank you for all the support and for helping me on so many occasions.

Thank you José Feijó for showing me new horizons, for all the support and guidance throughout this year.

Thank you Anabela Bernardes da Silva for all the encouragement, support and kindness during the last years. I am deeply grateful for the opportunity you gave me to develop my first laboratory project during my undergraduate studies.

To all the members of Liam Dolan's lab: thank you so much for making this year so enjoyable, for your precious help, input and friendship. In particular, thank you Thomas Tam for teaching me how to work with *Physcomitrella patens* and for your guidance during this year.

Thank you Lars Østergaard and Evelyn Koerner for the great collaboration developed in the *PpHOL* project.

To all my friends and family: Obrigado pelo carinho e amor que me transmitem em todos os momentos, vocês são essenciais na minha vida. Pai e mãe, amo-vos.

Filipa, you make it beautiful to live. Amo-te.

I dedicate this work to all of you.

The work illustrated in this thesis is supported by the European Union – Marie Curie – Integrated Training Network (PLANTORIGINS).

Abbreviations

bHLH	basic helix-loop-helix
bp	base pair
CDS	coding sequence
DNA	deoxyribonucleic acid
IAA	indole-3-acetic acid
kb	kilobase pair
LRL	LjRHL-LIKE
ML	maximum likelihood
N	sample number
NAA	1-naphtaleneacetic acid
PCIB	*p*-chlorophenoxyisobutric acid
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase PCR
RNA	ribonucleic acid
RSL	RHD SIX-LIKE
s.e.m.	standard error of the mean
TIBA	2,3,5-Triiodobenzoic acid
WT	wild type

Abstract

The evolution of developmental and physiological processes in plants made possible the colonization of the terrestrial environment. The development of a rooting system was critical for the spread of plants on land. Rhizoids and caulonema are cells with rooting function that are present in early diverging groups of land plants, such as mosses. In *Physcomitrella patens* the development of these cells is controlled by auxin, which positively regulates *PpRSL* (*Physcomitrella patens* RHD SIX-LIKE) Class I genes. The closest relatives of *PpRSLs* in higher plants control root hair development. In higher plants *LRL* (LjRHL-LIKE) genes also control root hair development but their function in *P. patens* is unknown. I show here that *PpLRL* genes are present in *P. patens* and are required for caulonema and rhizoid development. In *A. thaliana*, *RSL* Class I genes positively regulate the expression of *AtLRL3*, and this gene is also positively regulated by auxin. I demonstrate that auxin-induced rhizoid and caulonema cell development in *P. patens* requires the activity of *PpLRL* genes, but unlike *A. thaliana* both *PpLRL* genes are negatively regulated by auxin. I also found that expression of *PpLRL* genes is independent of *RSL* Class I. These findings suggest that there exists a gene network that is conserved between *A. thaliana* and *P. patens*, but the interactions between its components are different in the two species. These different topologies observed in the network that controls the development of rooting systems may explain the diversity observed in land plant rooting systems.

I also generated a knock-out mutant, *Pphol*, which will be used to understand the role and evolution of methyl halides production in land plants. Altogether, this work supports the hypothesis that the evolution of novel developmental and physiological processes was partly driven by the reutilization of ancient developmental and physiological mechanisms.

Key words: Basic helix-loop-helix transcription factor, rooting system evolution, *Physcomitrella patens*, auxin, *Harmless to Ozone Layer*

Resumo

A conquista de ambientes terrestres por parte das plantas constituiu um importante marco na História da Terra. Este evento teve impactos dramáticos nos ciclos atmosféricos e geoquímicos até aos dias de hoje. O desenvolvimento e a evolução de sistemas radiculares tornaram possíveis a conquista e adaptação das plantas a um ambiente terrestre. As primeiras plantas terrestres eram bastante semelhantes aos briófitos atuais e o seu sistema radicular era composto essencialmente por filamentos multicelulares chamados rizóides. O sistema radicular nas plantas superiores é constituído por pêlos radiculares, projeções unicelulares tubulares que cobrem a epiderme da raiz.

Recentemente descobriu-se que um par de fatores de transcrição beta helix-loop-helix (bHLH), denominados Root Hair Defective Six-Like (RSL) Classe I, controla o desenvolvimento de caulonema (células com funções radiculares) e rizóides no musgo *Physcomitrella patens* e o desenvolvimento de pêlos radiculares em *Arabidopsis thaliana*. Este facto demonstra que o mecanismo genético que controla o desenvolvimento de sistemas radiculares está presente nas plantas terrestres desde a existência do ancestral comum entre *P. patens* e *A. thaliana* há cerca de 443 milhões de anos. A regulação da rede transcricional que controla o desenvolvimento de pêlos radiculares em angiospérmicas é mais estudada e compreendida do que aquela que controla o desenvolvimento de caulonema e rizóides em briófitos. Para além dos fatores de transcrição RSL Classe I, outros factores de transcrição bHLH como os Lj-Root Hairless Like (LRL) promovem o desenvolvimento de pêlos radiculares em angiospérmicas.

A auxina é uma fitohormona que induz o desenvolvimento de pêlos radiculares em angiospérmicas e o desenvolvimento de rizóides em musgo. É sabido que a auxina não controla a expressão dos genes *AtRSL* Classe I mas controla positivamente a expressão do gene *AtLRL3* que por sua vez promove o desenvolvimento de pêlos radiculares em *A. thaliana*. Em *P. patens*, ao contrário do que ocorre em *A. thaliana*, a expressão dos genes *RSL* Class I, que controlam o desenvolvimento de rizóides, é controlada positivamente pela auxina. O genoma do musgo *P. patens* contém dois genes *LRL*, contudo a sua função em *P. patens* é desconhecida. Uma vez que os genes *RSL* Classe I funcionam tanto no desenvolvimento de rizoides em *P. patens* como no desenvolvimento de pêlos radiculares em *A*. *thaliana*, é postulado que os genes *LRL* funcionem também no desenvolvimento de rizóides no musgo *P. patens* à semelhança do que ocorre em *A. thaliana* (onde promovem o desenvolvimento de pêlos radiculares). Demonstrando que os genes *LRL* controlam o desenvolvimento de rizóides em *P*. *patens*, é possível inferir que mais do que um conjunto

de genes está presente na rede genética regulatória que controlou o desenvolvimento dos sistemas radiculares das primeiras plantas terrestres.

De forma a caracterizar a função dos genes *LRL* em *P*. *patens*, foi gerada uma análise filogenética de forma a inferir as relações filogenéticas entre os genes *LRL* presentes nos genomas de *A*. *thaliana* e *P*. *patens*. O genoma de *A*. *thaliana* contém cinco genes *LRL*, três dos quais controlam o desenvolvimento de pêlos radiculares (*AtLRL1*, *AtLRL2* e *AtLRL3*), e o genoma de *P*. *patens* contém dois genes *LRL* (*PpLRL1* e *PpLRL2*). Através da análise filogenética foi possível determinar que os genes *LRL* presentes em *P*. *patens* e os genes *AtLRL1*, *AtLRL2* e *AtLRL3* derivam do mesmo ancestral, dando suporte à hipótese de que *PpLRL1* e *PpLRL2* controlam o desenvolvimento de células com função radicular em *P*. *patens*. Para caracterizar a função de ambos os *LRL*, foi realizada uma análise fenotípica em mutantes que não contêm cada um dos genes: (*Pplrl1* e *Pplrl2*); e que sobre-expressem cada um dos genes: (*35S::PpLRL1* e *35S::PpLRL2*). Esta análise revelou que tanto *PpLRL1* e *PpLRL2* são necessários para o desenvolvimento de caulonema e rizóides (os dois tecidos com função radicular em *P*. *patens*). Os mutantes que não continham cada um dos genes desenvolvem filamentos de caulonema mais curtos e a coloração dos rizóides é afetada, sendo que em vez de desenvolverem rizóides com uma coloração acastanhada, desenvolvem rizóides mais pálidos comparados com as plantas *wild type*. Por outro lado, os mutantes *35S::PpLRL1* desenvolvem rizóides mais compridos e com uma coloração acastanhada mais demarcada quando comparados com as plantas *wild type*. Desta forma é possível afirmar que os componentes que compõem a rede regulatória genética que controla o desenvolvimento de sistemas radiculares nas plantas terrestres estão conservados desde a divergência entre *P. patens* e *A. thaliana*.

Uma vez que a expressão de *AtLRL3* é positivamente regulada pelos genes *RSL* Classe I em *A.thaliana*, e tendo em conta que os genes *RSL* Classe I também controlam o desenvolvimento de rizóides em *P*. *patens*, é postulado que a expressão de *PpLRL1* e *PpLRL2* é regulada pelos genes *RSL* Classe I em *P. patens*. De forma a verificar esta hipótese, os níveis de expressão dos genes *PpLRL1* e *PpLRL2* foram quantificados através de qRT-PCR no duplo mutante *Pprsl1 Pprsl2* (mutante sem os genes *RSL* Classe I). Espantosamente, tanto a expressão do gene *PpLRL1* como a do gene *PpLRL2* não é afetada no duplo mutante *Pprsl1 Pprsl2*. Este facto demonstra que, ao contrário do que acontece em *A. thaliana*, a expressão dos genes *LRL* é independente dos genes *RSL* Classe I em *P*. *patens*.

A auxina desempenha um papel crucial no desenvolvimento de pêlos radiculares em angiospérmicas e no desenvolvimento de células com função radicular no musgo *P. patens*.

É sabido que a expressão do gene *AtLRL3* é positivamente controlada pela auxina, regulando assim o desenvolvimento de pêlos radiculares através da sua ação na expressão do gene *AtLRL3*. De forma a compreender se a ação da auxina no desenvolvimento de rizoides e caulonema requer a ação dos genes *PpLRL1* e *PpLRL2*, os mutantes *Pplrl1* e *Pplrl2* foram tratados com auxina e comparados com os respectivos controlos (mutantes sem qualquer adição de auxina exógena). O tratamento de auxina em ambos os mutantes não foi capaz de despoletar o desenvolvimento de caulonema e rizóides observado em plantas *wild type*, revelando que o desenvolvimento mediado pela auxina de células com função radicular em *P. patens* requer a função dos genes *PpLRL1* e *PpLRL2*.

Tendo em conta que a expressão do gene *AtLRL3* é positivamente regulada pela auxina, e que o mecanismo de desenvolvimento de caulonema e rizóides induzido pela auxina requer a função de ambos os genes *PpLRL1* e *PpLRL2*, é postulado que a auxina desempenha um papel crucial na regulação da expressão destes genes. Para verificar esta possibilidade, o nível de expressão dos genes *PpLRL1* e *PpLRL2* foi quantificado através de qRT-PCR em plantas tratadas com auxina exógena e plantas não tratadas. Ao contrário do que seria expectável, tanto a expressão de *PpLRL1* como a de *PpLRL2* é negativamente regulada pela auxina. Este resultado demonstra que, embora os componentes da rede genética regulatória que controla o desenvolvimento de células com função radicular em plantas terrestres estejam conservados, a topologia dessa mesma rede genética regulatória é diferente entre *P*. *patens* e *A. thaliana*. Estas diferentes interações na mesma rede genética regulatória podem justificar a diversidade morfológica observada nos sistemas radiculares em plantas terrestres.

Nesta tese de mestrado é também demonstrada a geração de um mutante através de recombinação homóloga em *P*. *patens*. O objectivo é criar um mutante cujo gene *Harmless to Ozone Layer* (*HOL*) seja retirado do genoma do musgo *P*. *patens* de forma a criar uma planta que não contenha este gene: *Pphol*. O gene *AtHOL* é uma metiltransferase responsável pela catálise de haloalcanos em *A*. *thaliana*. A função dos haloalcanos não é bem conhecida em *A*. *thaliana*, mas é sabido que muitas espécies de plantas são capazes de produzir e emitir haloalcanos. O musgo *P*. *patens* é uma das plantas que produz e emite haloalcanos e sabe-se que o seu genoma contém um gene *HOL*, contudo a sua caracterização nunca foi realizada. De modo a caracterizar a função deste gene em *P. patens*, a emissão de haloalcanos será analisada no mutante *Pphol*, cuja geração é descrita nesta tese, e comparada com as plantas *wild type*. Se se verificar que este gene é necessário para a produção de haloalcanos em *P*. *patens*, é possível inferir que o mecanismo de síntese de haloalcanos está conservado nas plantas terrestres desde a divergência de *P. patens* e *A*. *thaliana*.

Juntamente com a análise dos fatores de transcrição PpLRL1 e PpLRL2, o uso do mutante *Pphol*, poderá ser essencial para a compreensão da evolução de mecanismos fisiológicos e de desenvolvimento em plantas terrestres. A evolução destes processos foi crucial para a adaptação e radiação das plantas no ambiente terrestre.

Palavras-chave: Fatores de transcrição basic helix-loop-helix, evolução de sistemas radiculares, *Physcomitrella patens*, Auxina, *Harmless to Ozone Layer*.

Table of contents

Chapter 1.
Introduction

Land plant evolution has been an important process that has been on-going since the conquest of the land by the ancestor of terrestrial plants over 470 million years ago (Kenrick and Crane, 1997; Berner, 2001; Kenrick and Davis, 2004). This pivotal event had dramatic impacts on the Earth system, bringing about significant changes in atmospheric and geochemical cycles (Igamberdiev and Lea, 2006; Lenton et al., 2012). The conquest of the land by plants was made possible by a series of developmental and physiological transformations. One of the features that made possible the colonization of the terrestrial environment was the evolution of a rooting system which allowed the plant to anchor itself to the substrate and to acquire nutrients from the soil. The first land plants had a bryophyte-like body with a dominant multicellular haploid (gametophyte) phase of the life cycle where rhizoids functioned as the rooting system. During land plant evolution there was an increase of the size and complexity of the multicellular diploid generation (sporophyte), which represents the predominant phase of the life cycle in higher plants. In the sporophyte of higher plants the rooting structures comprise roots and hairs that emerge from their surface (root hairs).

Genome sequencing efforts carried out in recent years have shown that basal plants such as mosses, with a complex multicellular gametophyte generation, have homologues of many genes that control the development of higher plants where the sporophyte is the dominant generation (Rensing et al., 2008). Indeed, the mechanism that controls the development of cells with a rooting function is conserved among land plants, where at least one closely related set of genes control both rhizoid development in the moss and root hair development in higher plants. However, the degree of conservation of the gene regulatory network that controls rhizoid and root hair development is unknown. The aim of this thesis is to characterise the role of a pair of genes in *Physcomitrella patens*, whose closest relatives control root hair development in Arabidopsis. *LRL* genes encode putative transcription factors that control root hair development in *Arabidopsis thaliana*, *Lotus japonicus* and *Oryza sativa*. The research reported in this thesis sets out to test the hypothesis that these genes control rhizoid development. If *LRL* genes control rhizoid development it demonstrates that more than one set of genes is conserved in the mechanism that controls rooting systems development.

1.1. The life cycle of *Physcomitrella patens*

Liverworts, mosses and hornworts are the most ancestral groups of land plants (Edwards et al., 1995; Kenrick and Crane., 1997). The fact that they represent the earliest diverging group of land plants and their simplicity of morphology and development, in comparison with higher plants, makes them a good system to address questions related to

the evolution of developmental and physiological processes in plants. The moss *Physcomitrella patens* has several additional characteristics that make it a suitable model plant for studies of the evolution of development. The complete genome sequence of *P. patens* has been determined (Rensing et al., 2008) and the high rate of homologous recombination allows knock-out mutants to be generated relatively easily by gene targeting (Schaefer and Zrÿd, 1997; Hiwatashi et al., 2001; Hobe et al., 2004; Kamisugi et al., 2005).

The life-cycle of the moss *P. patens*, like all bryophytes, displays alternation of generations. The dominant generation of the life cycle of *P. patens* is the gametophyte and the sporophyte only develops at the apex of the gametophores and forms only a small part of the adult plant (Figure 1.1). A haploid cell, the spore, germinates and generates primary a filamentous stage of the gametophyte called protonema. The protonema consists of chloronemata, which is defined by small cells with many chloroplasts and can differentiate into caulonemata, defined by its longer cells and the lack of chlorophyll. The switch from a two-dimensional network of filaments to a three-dimensional leafy shoot (gametophore) occurs with the development of a bud that contains the gametophore apical cell, usually formed by a caulonema secondary branch. This bud develops into a gametophore from which rhizoids develop. The moss *P. patens* is a monoicous species; both gametangia are present in the same plant, usually in the apex of the gametophore where the archegonium (female structure) and antheridium (male structure) are formed under low temperatures and short day conditions. The spermatozoids (male gametes) need a moist environment to allow them to swim to the egg cell (female gamete) where fertilization occurs. After fertilization, the zygote develops into a small diploid sporophyte where meiosis happens to produce haploid spores. The entire life cycle can be completed in less than 12 weeks under optimal conditions (Schaefer and Zrÿd, 2001).

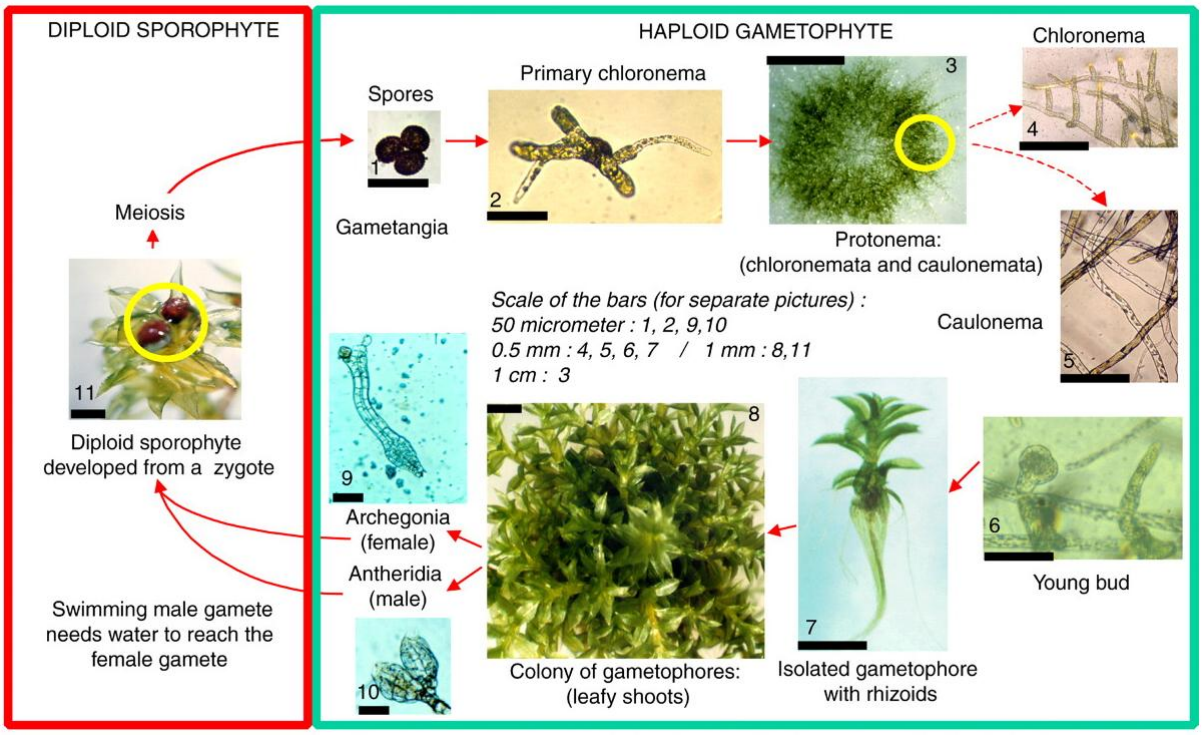


Figure 1.1 – The life cycle of *Physcomitrella patens* (adapted from Schaefer and Zrÿd, 2001).

1.1.1. Protonema development

Protonema comprises two different types of tip growing cells: chloronema and caulonema (Goode et al. 1992, Menand et al., 2007a). Chloronemata are the first type of cells formed after spore germination, while caulonemata differentiate from chloronema cells beginning several days after germination. These two types of cells differ morphologically. Chloronema cells contain many large chloroplasts and grow slowly in comparison to caulonema cells, which contain few chloroplasts and have a high growth rate (Duckett et al., 1998; Menand et al., 2007a). In addition, caulonemata have an oblique cell wall whereas chloronemata have a transverse cell wall perpendicular to the axis of growth (Menand et al., 2007a). These morphological characteristics are related with their respective function: chloronemata are essentially photosynthetic cells while the major roles of caulonemata cells are substrate colonization and nutrient acquisition (Duckett et al., 1998).

Protonema development, in particular, chloronema-to-caulonema transition, is controlled by endogenous and exogenous factors. Nutrient source and abundance both influence protonema development. The use of ammonium tartrate as a nitrogen source or a nutrient-rich substrate induces the development of chloronema whereas a nutrient-poor

substrate causes the preferential development of caulonema filaments (Jenkins and Cove, 1983). Energy (carbon) source also influences the development of protonema (Thelander et al., 2005). The provision of external glucose induces caulonemata formation whereas low energy conditions stimulate chloronemata growth (Thelander et al., 2005). This observation is concomitant with the function of caulonema and chloronema: under low energy conditions, chloronema growth is stimulated leading to higher rates of photosynthesis; but when in high energy conditions the moss can afford to produce caulonema and consequentially increase the size of the colony. Light is also a major factor that influences protonema branching and caulonema differentiation (Imaizumi et al., 2002). Both blue and red light induce the formation and position of protonemal side branches. Moreover, cryptochrome blue light signals inhibit auxin-induced caulonema differentiation and the expression of auxin inducible genes (Imaizumi et al., 2002), suggesting a close interlink between auxin and blue light.

Auxin is crucial for the differentiation of caulonema from chloronema. Auxin treatment induces the transition from chloronema to caulonema, while treatment with the anti-auxins PCIB (*p*-chlorophenoxyisobutric acid) or TIBA (2,3,5-Triiodobenzoic acid) suppresses caulonema differentiation, suggesting that auxin is a positive regulator of the chloronema-to-caulonema differentiation (Johri and Desai, 1973; Sood and Hackenberg, 1979; Bopp, 1980; Jang and Dolan, 2011).

1.1.2. Rhizoid development

In *P. patens*, rhizoids are brown-pigmented multicellular filaments that develop from epidermal cells of a gametophore stem. There are two types of rhizoids in the moss *P. patens*, the basal and the mid-stem rhizoids, which are morphologically indistinguishable (Sakakibara et al., 2003). Basal rhizoids are formed by all epidermal cells in the base of the gametophore, while the mid-stem rhizoids develop from an epidermal cell below an adult leaf (Sakakibara et al., 2003). The function of rhizoids in basal plants is very similar to those of root hairs in higher plants, which are anchoring the plant to the substrate and the uptake of water and nutrients (Jones and Dolan, 2012).

Similar to caulonema, rhizoids elongate by tip growth, produce oblique cross walls and possess few or no chloroplasts (Duckett et al., 1998). There are several steps in the development of a rhizoid filament. The protrusion of an epidermal cell from the gametophore is the first step in rhizoid development. This protrusion elongates by tip growth and divides into an apical cell and a subapical cell, separated by an oblique cross wall. The apical cell elongates and undergoes further divisions to form a filamentous rhizoid with several cells

(Sakakibara et al., 2003). In contrast to caulonema, most rhizoids do not usually form branches.

The effect of auxin on rhizoid development is well known. Exogenous auxin treatment results in the development of an increased number of rhizoids (Ashton et al., 1979; Sakakibara et al., 2003). The molecular mechanism that controls rhizoids development has been a subject of study in recent years, although as yet there is no comprehensive understanding of the transcriptional regulatory network that controls rhizoid development, in contrast to the extensive understanding of the one that controls root hair development in angiosperms. Nevertheless, some auxin-inducible transcription factors are known to be necessary for the normal development of rhizoids. These major findings are described below.

1.1.3. Auxin involvement in moss development

Plant hormones play a role in virtually every developmental process in plants. Auxin is one of the most-studied plant hormones in a variety of species, and it controls many aspects of the plant development, such as root initiation, root hair elongation, induction of apical dominance and vascular tissue differentiation in higher plants (Jacobs, 1951; Pitts et al., 1998; Booker et al., 2003; Knox et al., 2003; Weijers and Jurgens, 2005; Yi et al., 2010). In the moss *P. patens*, auxin positively regulates the cytokinin-dependent induction of the gametophore apical cell, the differentiation of chloronema to caulonema and the formation of rhizoids (Ashton et al., 1979; Sakakibara et al., 2003; Jang and Dolan, 2011; Jang et al., 2011; Aoyama et al., 2012). The use of model plants such as *Arabidopsis thaliana* and *P. patens*, in which it is possible to make genetic and molecular biological studies was crucial for the expansion of our knowledge of the biosynthesis, signalling pathway and biological roles of auxin.

The completed genome sequences of *P. patens* suggest that the genes required for auxin biosynthesis and signal perception in *A. thaliana* are also present in *P. patens* (Rensing et al., 2008). *A. thliana* SHI/STY proteins positively regulate auxin biosynthesis genes (Sohlberg et al., 2006; Ståldal et al., 2008; Eklund et al., 2010a). Two homologues of these genes (*PpSHI1* and *PpSHI2*) were found in the *P. patens* genome, which also regulate auxin concentration in moss (Eklund et al., 2010b). Loss-of-function of these two genes resulted in a decreased auxin concentration, whereas overexpression of *PpSHI1* led to an increased auxin concentration, suggesting that the mechanism has been conserved since *P. patens* and *A. thaliana* last shared a common ancestor (Eklund et al., 2010b).

The auxin signalling pathway in higher plants is mediated by the regulation of AUX/IAA proteins degradation (Dharmasiri and Estelle, 2004). These proteins interact with auxin response factors (ARFs) which positively or negatively regulate the transcription of a set of genes. The result of this interaction is the inactivation of the ARFs-regulated gene expression of target genes, i.e. the inactivation of genes positively controlled by ARFs or the activation of genes negatively controlled by ARFs (Ulmasov et al., 1997; Rouse et al., 1998 Liscum and Reed, 2002). Auxin controls the concentration of AUX/IAA proteins by regulating the degradation of these proteins in the proteasome complex SCF^{TIR1}, leading to a decreased interaction between ARFs and AUX/IAA and consequently to the activation of the ARFs-regulated gene expression of target genes (Gray et al., 2001; Ramos et al., 2001). Recently, mutations in *AUX/IAA* genes were identified in *P. patens* auxin resistant mutants that show caulonema and rhizoid defective phenotypes (Prigge et al., 2010). In this study it was also shown that moss AUX/IAA proteins interact with AtTIR1 protein, and silencing *PpAFB* (homologue of the Arabidopsis TIR1) resulted in an auxin resistant mutant phenotype, suggesting that the auxin signalling pathway has been conserved between bryophytes and higher plants during land plant evolution.

There is evidence of polar auxin transport in caulonemata, rhizoids and sporophytes of moss (Rose and Bopp 1983; Bopp and Atzorn, 1992; Poli et al., 2003; Fujita et al., 2008). However there is no evidence of polar auxin transport in the leafy shoot of *P. patens* (Fujita et al., 2008). The use of a fusion between *Glycine max* GH3 promoter (which is responsive to auxin) and the reporter gene beta-glucoronidase (GUS) in *P. patens* shows that the sites of auxin response coincide with the expression of *SHI* genes (Bierfreund et al., 2003; Ludwig-Müller et al., 2009; Eklund et al., 2010). In addition, no PIN-type proteins responsible for efflux of auxin from cell to cell were found in the *P. patens* genome sequence (Mravec et al., 2009). Altogether, these evidences suggest that is not polar auxin transport but local auxin biosynthesis that plays a major role in the formation of auxin maxima in *P. patens*.

The expression of a variety of genes in *P. patens* is modulated by auxin. In particular, the expression of transcription factors that control rhizoid and caulonema differentiation are positively regulated by auxin (Sakakibara et al., 2003; Jang et al., 2011). This indicates that auxin induces rhizoids and caulonema differentiation by positively regulating genes required for the development of these cells.

1.2. Transcriptional regulation of root hair development in *Arabidopsis thaliana*

Root hair development is one process in higher plants that is regulated by auxin (Knox et al., 2003; Yi et al., 2010). Root hairs are tip-growing projections that grow from cells of the root epidermis (Dolan et al., 1994). They are crucial for nutrient and water uptake, soil weathering, anchorage and interaction with soil microorganisms (Peterson and Farquhar, 1996; Gahoonia et al., 1997; Gahoonia and Nielsen, 1998; Datta et al., 2010). There are three developmental steps for root hair development in *A. thaliana*: cell fate determination, root hair initiation and elongation by tip growth.

The cell fate specification is regulated by an intercellular gene regulatory network that includes the transcription factors *GLABRA 2 (GL2)*, *TRANSPARENT TESTA GLABRA (TTG)*, *GLABRA 3 (GL3)*, *ENHANCER OF GLABRA 3 (EGL3)* and *WEREWOLF (WER)* that control non-hair cell fate, and *CAPRICE (CPC), TRYPTYCHON (TRY)* and *ENHANVER OF CPC (ETC)* that control hair cell fate (Galway et al., 1994; Rerie et al., 1994; Cristina et al., 1996; Masucci et al., 1996; Wada et al., 1997; Lee and Schiefelbein, 1999; Walker et al., 1999; Schellmann et al., 2002; Wada et al., 2002; Bernhardt et al., 2003; Kirik et al., 2004; Simon et al., 2007). The interaction between complexes of these genes and the mobility of some of these transcription factors regulate the cell patterning in the *A. thaliana* root epidermis which result in files of hair cells and non-hair cells along the root axis (Lee and Schiefelbein, 2002).

Once root hair cell fate has been specified, root hairs initiate from the surface of hair cells and are controlled by different genes than those which control cell fate. Class I RSL proteins, ROOT HAIR DEFECTIVE 6 (AtRHD6) and RHD-SIX-LIKE 1 (AtRSL1), are members of subfamily VIIIc of basic helix-loop-helix (bHLH) transcription factors that function after the epidermis cell fate has been specified in the root of *A. thaliana* (Masucci and Schiefelbein 1994; Menand et al., 2007b). *A. thaliana* roots homozygous for loss-of-function mutations in both of these genes do not develop root hairs in normal growth conditions (Menand et al, 2007b). In addition, it was shown that Class I *RSL* genes are only expressed in hair cells and their expression is positively regulated by *CPC* whereas *WER*, *TTG* and *GL2* negatively regulate the expression of Class I *RSL* genes (Menand et al, 2007b). Class I RSL induces the expression of other transcription factors, such as genes encoding bHLH transcription factors, to control root hair expansion and differentiation (Karas et al., 2009; Yi et al, 2010; Bruex et al., 2012). Taken together these data indicate that Class I *RSL* genes are positive regulators of root hair development in *A. thaliana*.

A number of bHLH transcription factors promote root hair growth and act downstream of Class I *RSL* genes (Yi et al., 2010; Bruex et al., 2012). *RHD-6-LIKE 4* (*RSL4*), a member of the subfamily VIIIc of the bHLH transcription factors, which belongs to Class II *RSL* genes, is a direct target of Class I RSL and regulates root hair expansion (Yi et al., 2010). Plants lacking the *RSL4* gene developed shorter root hairs while plants overexpressing this gene showed longer root hairs when compared to the wild type, suggesting that RSL4 is required and sufficient for root hair elongation (Yi et al., 2010). Another bHLH that acts downstream of Class I RSL and it is necessary for normal root hair growth is LjRHL1-LIKE 3 (LRL3). This protein belongs to the subfamily XI of bHLH transcription factors and loss-of-function mutants exhibit shorter root hair cells when compared to the wild type (Karas et al., 2009; Bruex et al., 2012).

Root hair cells differentiation is controlled by auxin (Knox et al., 2003). Interestingly, the transcription of Class I *RSL* genes is not regulated by auxin and the phenotype of loss-of-function mutants can be rescued by applying exogenous auxin to the media, suggesting that auxin regulates root hair elongation downstream of Class I RSL (Yi et al., 2010; Jang et al., 2011). Indeed, microarray data, gene expression analysis and auxin treatment in mutant backgrounds suggest that auxin promotes root hair growth by positively regulating the expression of *RSL4* and *LRL3*, transcription factors required for root hair elongation, as well as other genes downstream of Class I RSL in *A. thaliana* (Yi et al., 2010; Bruex et al., 2012).

1.3. *RSL* genes and caulonema and rhizoid development

The genetic mechanism that controls the development of cells with a rooting function in moss is less understood than it is in *A. thaliana*. Recent studies shed a light on the transcriptional regulation of caulonema and rhizoid development in *P. patens*. There are seven *RSL* genes in the genome of the moss *P. patens* (Menand et al., 2007b; Jang et al., 2011). Two of them are Class I *RSL* genes (*PpRSL1* and *PpRSL2*) while the other five constitute the Class II *RSL* (*PpRSL3* – *PpRSL7*) genes (Menand et al., 2007b; Jang et al., 2011). Mutants lacking both *PpRSL1* and *PpRSL2* develop few and shorter rhizoids and no caulonema, which indicates that Class I RSL is necessary for both caulonema and rhizoid development (Menand et al., 2007b). Given the similarity not only in function but also in morphology between root hairs, rhizoids and caulonemata and the fact that homologous genes control their development, it was hypothesized that the RSL mechanism might be conserved between *P. patens* and *A. thaliana*. To address this question, the *PpRSL1* gene was introduced in the *Atrhd6/rsl1* mutant background in Arabidopsis and remarkably it resulted in the normal development of root hairs (Menand et al., 2007b). This experiment shows that the same gene regulatory mechanism has been conserved in land plant evolution

since the divergence of bryophytes and higher plants to control the differentiation of cells with a rooting function (Menand et al., 2007b).

Overexpression of both *PpRSL1* and *PpRSL2*, transform most cells in a bud (structure that gives rise to the gametophore) into rhizoids, creating a mass of rhizoids instead of the usual shoot-like gametophore, which indicates that Class I RSL is sufficient for rhizoid development in *P. patens* (Jang et al., 2011). In addition, *PpRSL1* and *PpRSL2* are expressed in cells that give rise to rhizoids, supporting the hypothesis that their expression is sufficient for rhizoid development (Jang et al., 2011).

In contrast to Class I *RSL* genes in Arabidopsis, the Class I *RSL* genes in *P. patens* are positively regulated by auxin (Jang and Dolan, 2011; Jang et al., 2011). It was shown that the application of exogenous auxin increased the expression levels of *PpRSL1* and *PpRSL2* (Jang and Dolan, 2011; Jang et al., 2011). Moreover, these genes are expressed in the cells that are relatively sensitive to auxin and auxin treatment of *Pprsl1/Pprsl2* double mutant did not increase rhizoids number or rescue the caulonema development compared with the untreated double mutant (Jang and Dolan, 2011; Jang et al., 2011). Altogether, these experiments showed that auxin induces Class I *RSL* genes to promote both rhizoid development and the transition of chloronema to caulonema (Jang and Dolan, 2011; Jang et al., 2011). Moreover, despite the different interactions between *RSL* genes and auxin in the control of rhizoid and root hair development, these data suggest that *RSL* genes and auxin are components of an ancient genetic regulatory network that controls rooting cells development that was present in the last common ancestor of mosses and angiosperms (Jang et al., 2011)

Class II RSL proteins only control protonema development, specifically, the transition of chloronema to caulonema (Pires, 2010). In addition, the interactions between *RSL* Class I and *RSL* Class II are different in Arabidopsis and *P. patens* (Yi et al., 2010; Pires, 2010). While the expression of *RSL* Class II genes is induced by Class I RSLs in Arabidopsis, the same does not occur in *P. patens*, suggesting a change in the topology of the transcriptional network between *RSL* Class I and *RSL* Class II, besides the level of interaction with auxin, during land plant evolution (Yi et al., 2010; Pires, 2010).

Despite the knowledge about the RSL mechanism, little is known about the wider transcriptional regulatory network that controls cells with rooting functions in moss. One goal of the study presented in this thesis is to characterize a putative downstream target of Class I RSL in the moss *P. patens* using phenotypic analysis of mutants, auxin treatment and gene expression analysis of the candidate genes to elucidate the level of conservation of the mechanism that controls the development of the rooting system in land plants.

1.4. Methyl halide production and HOL function

Methyl halides are compounds derived from methane (CH_4) with one or more halogen atoms (F, Cl, Br, or I), which are emitted naturally from oceans, wood-rotting fungi, salt marshes and predominately in tropical and subtropical forests (Khalil and Rasmussen, 1999; Lobert et al., 1999; Yokouchi et al., 2002; Harper et al., 2003; Rhew et al., 2003; Saito and Yokouchi, 2006). Methyl chloride (CH_3Cl) and methyl bromide (CH_3Br) are the primary carriers of chlorine and bromine, respectively, to the stratosphere where their catalytic reactions play a major role in the destruction of the ozone layer (Penkett et al., 1980), whereas methyl iodide (CH_3I) influences aerosol formation and ozone loss in the troposphere (Chameides and Davis, 1980; Alicke et al., 1999; O'Dowd et al., 2002).

Plants produce and emit methyl halides, and the production of these compounds is catalysed by a methyltransferase protein encoded by the *HARMLESS TO OZONE LAYER* (*HOL*) gene in *A. thaliana* (Rhew et al., 2003; Nagatoshi and Nakamura, 2009). Mutant plants with loss-of-function of the *HOL* gene show almost no production of methyl halides proving that HOL is required for the production of methyl halides in *A. thaliana* (Rhew et al., 2003). The biological function of HOL and methyl halides is not clear in plants but a recent study suggests that methyl halides are involved in defence against phytopathogens (Nagatoshi and Nakamura, 2009).

The genome of *P. patens* encodes one HOL protein and measurements of methyl halides emission in *P. patens* show that the moss emits methyl halides (Lars Østergaard, personal communication, August 3, 2012). Moreover, microarray data and gene expression analysis show that the gene is expressed both in protonema and gametophore (Lars Østergaard, personal communication, August 3, 2012), suggesting that the protein may function in both the protonema and gametophore.

In this thesis I describe the generation of a *Pphol* knock-out mutant that can be used to test if PpHOL is required for methyl halide production and to determine if this protein has any other function in the moss *P. patens*. Together with the complementation of the *Athol* mutant with the *PpHOL* gene, it will allow us to determine if the mechanism of methyl halides synthesis is conserved among land plants.

Chapter 2. Material and Methods

2.1. Phylogenetic analysis

Coding sequences from the gene model databases of *Arabidopsis thaliana* and *Physcomitrella patens* were retrieved from Phytozome v8.0 (http://www.phytozome.net/). Amino acid sequences of the bHLH and LRL domains were aligned using T-Coffee (http://tcoffee.crg.cat/) and posterior aligment editing was done in the program BioEdit version 7.1.3 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The Jones, Taylor, and Thornton (JTT) model was selected as the best-fitting amino acid substitution model with the Akaike information criterion implemented in ProtTest (Abascal et al. 2005). ML (Maximum likelihood) analysis was performed with the program Seaview version 4.4.0 (http://pbil.univ-lyon1.fr/software/seaview.html) using the JTT model of amino acid substitution, an estimated gamma distribution and across site rate variation (JTT+G+I). The Bayesian analysis was performed with MrBayes version 3.1.2 (http://mrbayes.csit.fsu.edu/): two independent runs were computed for 10 million generations, at which point the standard deviation split frequencies was less than 0.01; one tree was saved every 100 generations, and 75% of the trees from each run were summarized to give rise to the final cladogram. Both ML and Bayesian tree were visualized using the program Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

2.2. Plant materials and growth conditions

The Grandsen wild type strain of *Physcomitrella patens* (Hedw.) Brunch and Schimp (Ashton et al., 1979) was used in this study. Moss sporophytes were collected after sporophyte induction, of three weeks old protonema on jiffies pots, at 17 °C illuminated with a light regime of 8h light / 16h dark in a plant growth cabinet (Sanyo MLR-351), and they were surface sterilised in 5% (v/v) sodium hypochlorite for 10 minutes, rinsed five times with sterile distilled water and crushed to release the spores into the water. Sterile spore suspensions were kept at 4°C in darkness for several months. The minimal medium used for spore germination and for protonema phenotypical analysis contained 0.8 g/L $CaNO_3 4H_2O$, 0.25 g/L $MgSO_4 7H_2O$, 0.0125 g/L $FeSO_4 7H_2O$, 0.055 mg/L $CuSO_4 5H_2O$, 0.055 mg/L $ZnSO_4 7H_2O$, 0.614 mg/L H_3BO_3, 0.389 mg/L $MnCl_2 4H_2O$, 0.055 mg/L $CoCl_2 6H_2O$, 0.028 mg/L KI, 0.025 mg/L $Ne_2MoO_4 2H_2O$, 7 g/L agar (Formedium cat#AGA03) and 1 mL KH_2PO_4 buffer pH 6.5. The KH_2PO_4/KOH buffer contained 25 g KH_2PO_4 per 100 mL; pH 6.5 was obtained by titrating with 4M KOH. The minimal medium used for rhizoid phenotypic analysis contained 5 g phytagel (Sigma cat#048K0017) instead of 7 g agar (Formedium cat#AGA03) per Litre. 35 mL minimal media was poured in 90 mm plastic plates and overlaid with autoclaved

cellophane disks (AA packaging, UK) for spore germination and protonema phenotypical analysis; 400 mL media was poured in transparent plastic tubes for rhizoid phenotypic analysis.

For spore germination, sterile spore suspension was inoculated onto plastic plates with minimal media; plates were closed with Micropore tape. Mosses were grown at 25°C in plant growth cabinets (Sanyo MLR-351), illuminated with a light regime of 16h light / 8h darkness. For routine protonema propagation, minimal medium was supplemented with 500 mg/L ammonium tartrate and 5 g/L glucose; protonema tissue was blended with a homogenizer (PowerGen 500, Fisher Scientific). Protonema cultures were subcultured every 5-7 days.

Physcomitrella patens *Pplrl1-3*, *Pplrl1-6*, *Pplrl2-2* and *Pplrl2-6* loss-of-function mutants were obtained by homologous recombination method and targeted insertions were confirmed by PCR (Thomas Tam, University of Oxford). The overexpressed lines *35S::PpLRL1-1*, *35S::PpLRL1-6*, *35S::PpLRL2-1* and *35S::PpLRL2-5* were also generated by gene targeting method based on homologous recombination targeting the '108 locus' (Thomas Tam). The *Pprsl1 Pprsl2* double mutant was previously described (Menand et al., 2007b).

2.3. RNA extraction, cDNA synthesis and qRT-PCR analysis

Total RNA was isolated from frozen plant tissue with the RNeasy Plant Mini Kit (Qiagen cat#74904), with subsequent DNase treatment using Turbo DNA-free Kit (Invitrogen AM1907). RNA was quantified with a Qubit 2.0 fluorometer (Invitrogen, USA). For cDNA synthesis, RNA was reverse transcribed with the SuperScript III First/Strand Synthesis System for RT-PCR using oligo(dT) (Invitrogen cat#180080-051). qRT-PCR analysis was performed with the SYBR Green PCR Master Mix (Applied Biosystems cat#4364344) in the Applied Biosystems 7300 Real-Time PCR System. Cycle conditions were as follows: 10 minutes incubation at 95 °C followed by 40 cycles of 15 seconds incubation at 95 °C and one minute at 60 °C; a data collection step was performed at the end of each cycle. A dissociation stage was performed at the end of the run to confirm the amplification of specific amplicons. Relative expression levels were calculated by the ΔΔCt method, using the GAPDH (PHYPA_226280) and Elongation Factor 1α (PHYPA_158916) for normalization (Appendix 1).

2.4. Auxin treatment

Auxin treatment of *P. patens* protonema was carried out by plating spores on solid minimal media supplemented with 0.1 μM α-naphtalene acetic acid (NAA) (Sigma cat#86-87-3) overlaid with cellophane disks and incubating for 23 days.

2.5. Microscopy and statistical analysis

Protonema, gametophore and rhizoids were imaged with a Leica DFC310 FX camera mounted on a Leica M165 FC stereo microscope using the imaging software Leia Application Suite. All measurements were performed in ImageJ version 1.46 (Abramoff et al., 2006). Protonema colony diameter was determined as the mean of three end-to-end distances with the origin at the centre of the colony. Distances from the tip of a filament to the first branch were measured from filaments protruding from the edges of colonies. Microsoft Excel™ 2011 was used for statistical analysis; statistical significance tests were calculated by t-test.

2.6. Physcomitrella patens transformation

To generate the loss-of-funtion moss *Pphol*, a gene targeting system based on homologous recombination was used. This system makes it possible to modify a specific site of the genome without changing or damaging the genome. The *PpHOL* knock-out construct used for homologous recombination of the *PpHOL* locus was generated and provided by Evelyn Koerner (John Innes Centre, UK). This construct, derived from pBHrev, contains an ampicillin resistance gene and a hygromycin resistance gene. In order for the desired homologous recombination (in the *PpHOL* locus) to occur, a 5' genomic DNA fragment (881 bp) starting from 1.5 kb upstream of the start codon of *PpHOL* CDS was inserted into *NcoI* and *SpeI* sites, and a 3' genomic DNA fragment (765 bp) starting from 0.5 kb downstream of the stop codon of *PpHOL* was insterted into *HindIII* and *XbaI* sites. The construct was double digested with *AscI* and *HindIII* in order to improve the transformation efficiency. The double digested fragment was introduced into moss protoplasts by Polyethylene glycol (PEG)-mediated transformation technique as described previously by Schaefer and Zrÿd (1997). Three plates of protonemal tissues grown on minimal media supplied with 500 mg ammonium tartrate and 5 g glucose for five days were collected. 25 mL driselase was added to the protonema followed by 40 minutes incubation with gentle mixing. For collecting protoplasts, samples were filtered with microspore sieve and 8.5% mannitol. Collected protoplasts were suspended in 3 mL MMM solution (8.5% mannitol, 15 mL $MgCl_2$ and pH 5.6 of 0.1% MES). 300 μL of protoplasts suspension was added into a 50 mL tube which already

had 15 μg of digested DNA construct. 35% PEG (300 μL) was added to the DNA and protoplast suspension. After a heat shock at 45 °C for five minutes, the protoplasts were left at room temperature for 10 minutes. Minimal medium modified with the omission of $CaCl_2$ and the addition 500 mg/L ammonium tartrate, 5 g/L glucose and 66 g/L mannitol was added to the protoplast suspension. After overnight incubation in the dark, the protoplasts were placed onto solid minimal media supplemented with 500 mg/L ammonium tartrate, 5 g/L glucose and 66 g/L mannitol, and a layer of top agar (1.4% agar (Sigma cat#A9799) and 8.5% mannitol) was added. Transformants were selected on Hygromycin B (25 μL/mL) for two rounds. Stable transformants were confirmed by PCR (Appendix 1).

Chapter 3. *LRL* function in rooting cells of *Physcomitrella patens*

The study of the transcriptional network that controls the development of rooting cells in higher plants and in bryophytes is important for the understanding of rooting system evolution in land plants. Many components of the transcriptional network that controls root hair development have been identified in *Arabidopsis thaliana* and their transcriptional interactions characterized (Bruex et al., 2012). Recently, key components of this network, *RSL* genes, were identified in *Physcomitrella patens* and shown to positively regulate rhizoid and caulonema development. This indicates that the mechanism that controls the development of rooting cells is conserved between *A. thaliana* and *P. patens* (Menand et al., 2007b; Jang and Dolan, 2011; Jang et al., 2011). *RSL* genes comprise two classes, *RSL* Class I and *RSL* Class II genes. *RSL* Class I genes are required for caulonema and rhizoid development in *Physcomitrella patens* and for root hair initiation in *Arabidopsis thaliana*. In *A. thaliana*, *RSL* Class I genes positively control the expression of a variety of genes that regulate root hair growth (Menand et al., 2007b; Yi et al., 2010; Bruex et al., 2012). One of these genes is *AtLRL3* (which belongs to bHLH subfamily XI) (Karas et al., 2009; Bruex et al., 2012). There are five *LRL* genes in the genome of *A. thaliana* and three (*AtLRL1*, *AtLRL2* and *AtLRL3*) regulate root hair development. However, only *AtLRL3* is positively regulated by *RSL* Class I genes (Karas et al., 2009; Bruex et al., 2012). The role of *LRL* genes in *P. patens* has not yet been demonstrated.

The aim of the research reported in this chapter is to characterise the role of the *LRL* genes in *P. patens*. Because *LRL* genes control root hair development and the genome of *P. patens* contains two *LRL* genes, it is hypothesized that these genes control caulonema and rhizoid development in *P. patens*.

The work presented in this chapter is the continuation of previous work already done in the laboratory. Previously, loss-of-function and overexpression mutant lines for *PpLRL1* and *PpLRL2* genes were generated by homologous recombination and the recombination events were confirmed by PCR (Thomas Tam, University of Oxford).

3.1. Results

3.1.1. *LRL* genes are present in *Physcomitrella patens*

In a previous study it was shown that *LRL* (bHLH subfamily XI) genes are present in *P*. *patens* (Pires and Dolan, 2010); however the phylogenetic relationship between *Arabidopsis thaliana* and *P. patens LRL* genes was not determined. There are five *LRL* genes in the *A. thaliana* genome: *AtLRL1*/*AtbHLH066, AtLRL2*/*AtbHLH069*, *AtLRL3*/*AtbHLH082*, *AtLRL4*/*AtbHLH007* and AtUNE12/AtbHLH059 and two *LRL* genes in the *P. patens* genome: *PpLRL1*/*PpbHLH012* and *PpLRL2*/*PpbHLH013*, which according to

microarray data are expressed broadly in the gametophyte tissues of the moss (Hruz et al., 2008).

To determine the evolutionary relationship between *Arabidopsis* and *Physcomitrella* LRL proteins, the alignment shown in Appendix 2 was used to calculate Bayesian (Fig. 3.1) and ML (Maximum likelihood) trees (Appendix 3). The results from the Bayesian analysis are concordant with those of the ML analysis. The two *Physcomitrella* LRL proteins are clustered within the *Arabidospsis* LRL proteins. AtLRL1, AtLRL2 and AtLRL3 are sister to PpLRL1 and PpLRL2 suggesting that these genes are derived from a common ancestral gene that existed sometime before 443 million years ago. The resolution of these two clades is low (53) which supports this conclusion and suggests that they may even form a single monophyletic group, although further analysis will be required before this can be ascertained. In conclusion, LRL proteins evolved before the divergence of mosses from angiosperms, over 443 million years ago, and AtLRL1, AtLRL2, AtLRL3, PpLRL1 and PpLRL2 are derived from a common ancestral gene.

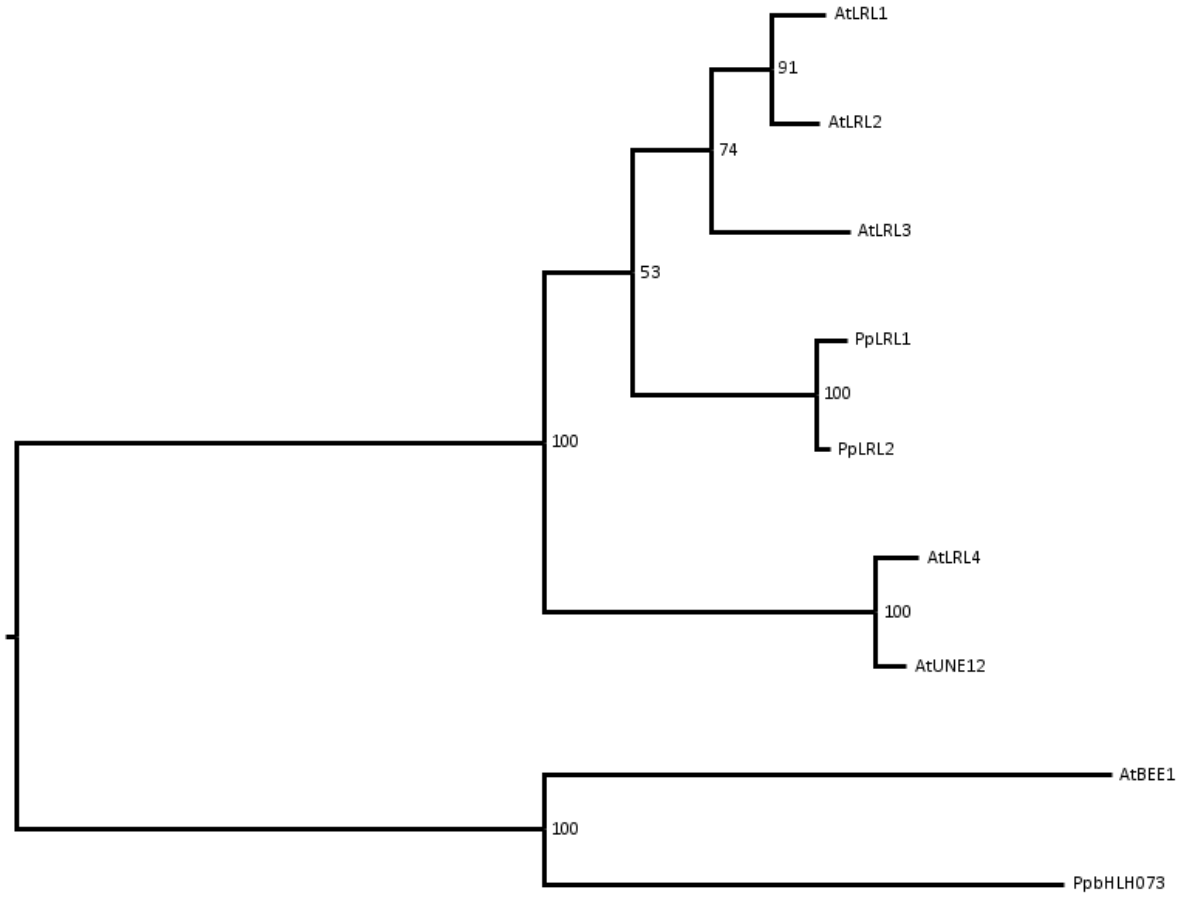


Figure 3.1 – *LRL* genes are present in the genome of *Physcomitrella patens*.

Bayesian phylogenetic tree of *A. thaliana* and *P. patens* LRL proteins. The tree was calculated using the alignment shown in Appendix 2 and rooted with two bHLH subfamily XII proteins (AtBEE1 and PpbHLH073). Posterior probability values are indicated in the nodes.

3.1.2. *PpLRL* are required for the development of caulonemata and gametophores

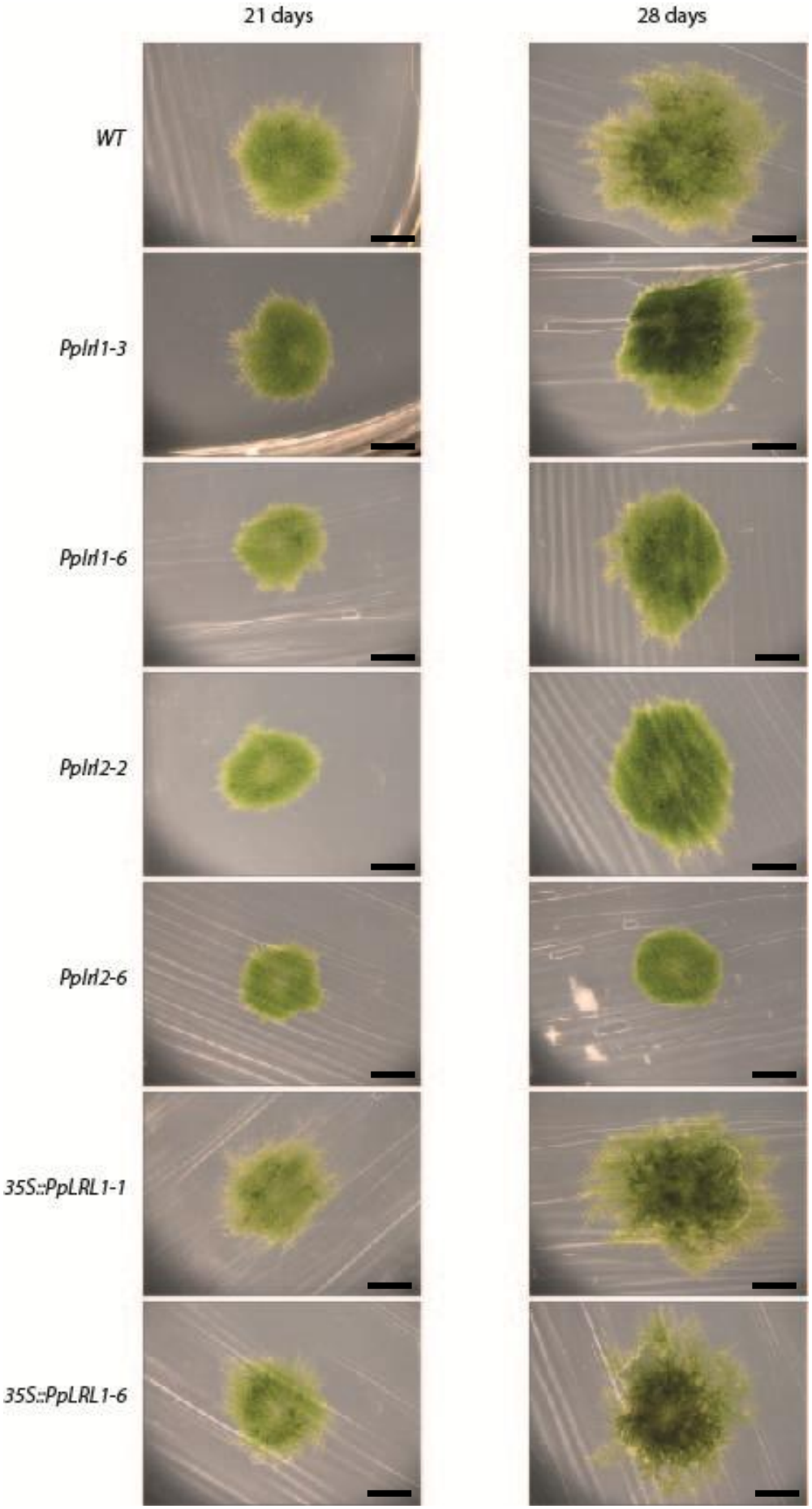
PpLRL positively controls caulonema development

LRL proteins control the development of root hairs and *AtLRL3* expression is positively regulated by RSL Class I proteins in *A. thaliana* (Karas et al., 2009; Bruex et al., 2012). It is known that RSL Class I control the development of rooting cells in both *A. thaliana* and *P. patens* (Menand et al., 2007b; Jang and Dolan, 2011; Jang et al., 2011). The function of LRL proteins in *P. patens* is still unknown. Since they control root hairs development in higher plants it is hypothesized that they might also control caulonemata and rhizoids development in moss. To test this hypothesis, a phenotypic analysis of protonema development was carried out in LRL loss-of-function and LRL overexpression lines.

To characterise protonema development in the loss-of-function (*Pplrl1-3, Pplrl1-6, Pplrl2-2 and Pplrl2-6)* and overexpression lines (*35S::PpLRL1-1, 35S::PpLRL1-6*, *35S::PpLRL2-1* and *35S::PpLRL2-5*), spores were germinated on minimal media overlaid with a cellophane disk and the development of protonema colonies was followed for four weeks. In WT moss, the inner region of the colony is composed predominantly of chloronema cells (shorter and filled with chloroplasts) whereas at the edges of the colony caulonema cells (long and with few chloroplasts) grow away from the centre (Fig. 3.2). At 21 and 28 days, both *Pplrl1* lines show a higher proportion of inner denser protonema than in WT plants, which is a result of the presence of fewer caulonema-like cells at the edge of the *Pplrl1* colonies than in WT (Fig. 3.2, 3.3 and 3.4). Fewer caulonema cells also develop in *Pplrl2* colonies compared to WT plants. To quantify this difference, measurements of the distance between the tip of filaments protruding from the edge of protonema colonies and their first side branch were taken to confirm the identity of the cells present at the edges of the colony. Because caulonema-like cells are longer than chloronema-like cells, the distance of the first side branch from the tip is longer in a filament composed by caulonema cells than by chloronema cells. As expected, the distance to the first side branch is shorter in *Pplrl1* and *Pplrl2* lines than WT plants (Fig. 3.5), which suggests that the edges of *Pplrl1* and *Pplrl2* are less caulonema-like than in WT and the diameter of colonies is reduced as a result of decreased caulonema development. These data suggest that *LRL* genes positively regulate caulonema development in *P. patens*.

To independently verify that *LRL* genes positively regulate caulonema development in *P. patens*, plants were transformed with gene constructs that overexpress each *LRL* gene. The distance of the first side branch from a filament tip is longer in all overexpressed lines

than in WT plants (with exception for *35S::PpLRL2-5* where this distance was shorter than wild type) (Fig. 3.5). It is possible that *PpLRL2* expression is repressed in the *35S::PpLRL2-5* background. Such co-repression remains to be verified. These data are consistent with the hypothesis that *LRL* genes positively regulate caulonema development. Nevertheless, the effect of *LRL* overexpression is subtle and the diameter of colonies that overexpress *LRL* genes is similar to wild type (Fig. 3.3). Taken together these results suggest that caulonema development is positively regulated by PpLRL1 and PpLRL2.


21 days
28 days
WT
PpIrl1-3
PpIrl1-6
PpIrl2-2
PpIrl2-6
35S::PpLRL1-1
35S::PpLRL1-6

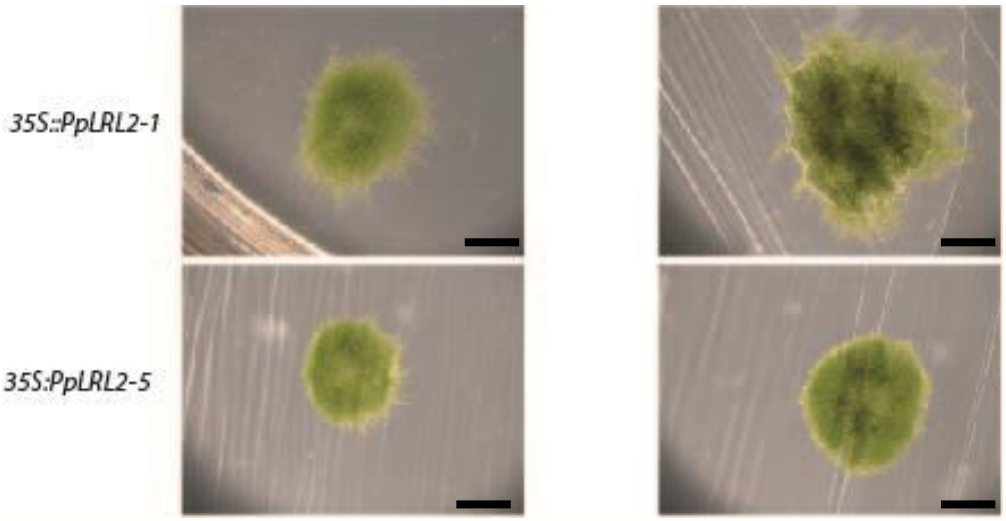


Figure 3.2 – *Pplrl1* and *Pplrl2* colonies are smaller than WT colonies.

Spores were germinated on minimal media overlaid with a cellophane disk. All loss-of-function mutants produce denser colonies with shorter filaments at the edges coming from the centre of the colony. All overexpression lines, except *35S::PpLRL2-5*, exhibit colonies similar to the WT colonies but in *35S::PpLRL1-1* and *35S::PpLRL1-6* it is observed more gametophores formed compared to the WT. Pictures were taken after 21 and 28 days. Scale bars indicate 2 mm.

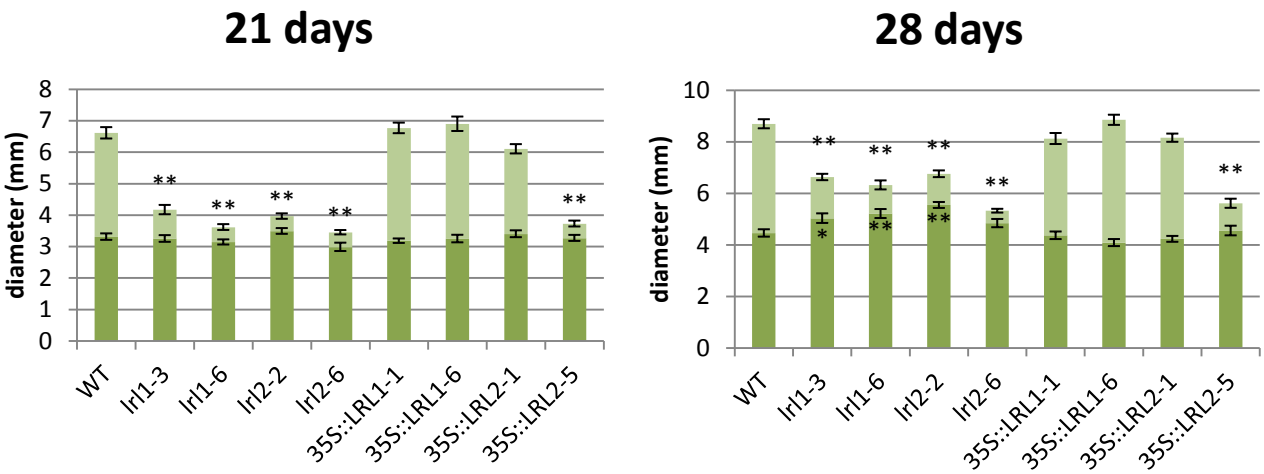
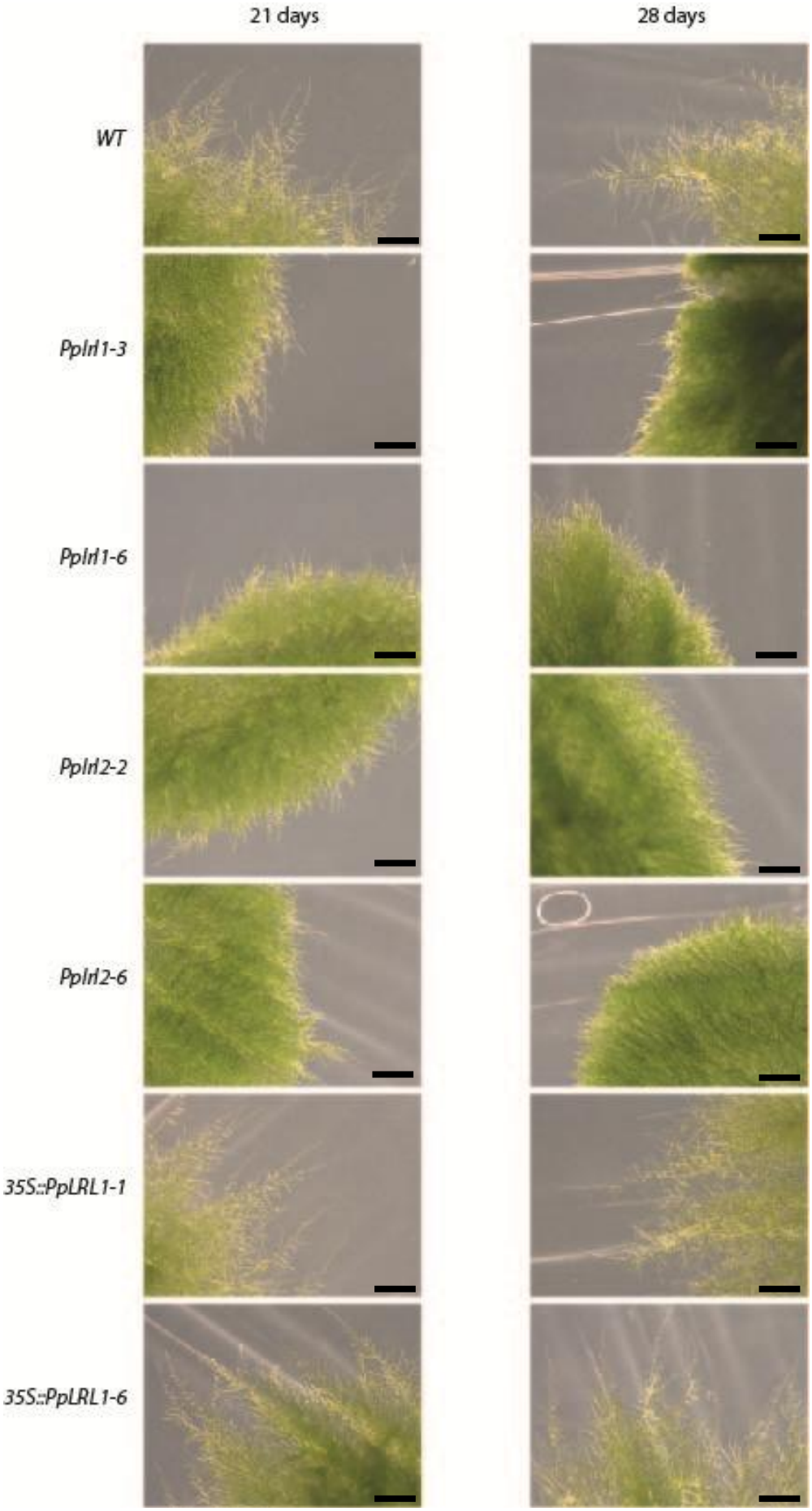


Figure 3.3 – Caulonema development is positively controlled by *PpLRL*

Spores were germinated on minimum media with cellophane disks; the diameter of protonema colonies (light green bars) and of the inner denser protonema region (dark green bars) were measured after 21 and 28 days. Colony diameter is reduced in *Pplrl1*, *Pplrl2* and *35S::PpLRL2-5* lines due to the development of few and shorter caulonema-like cells. Error bars indicate s.e.m., n=15. Asterisks indicate values that are statistically significantly different from WT ($p<0.05$ and $p<0.01$ (t-test) for single and double asterisks respectively).


21 days
28 days
WT
PpIrl1-3
PpIrl1-6
PpIrl2-2
PpIrl2-6
35S::PpLRL1-1
35S::PpLRL1-6

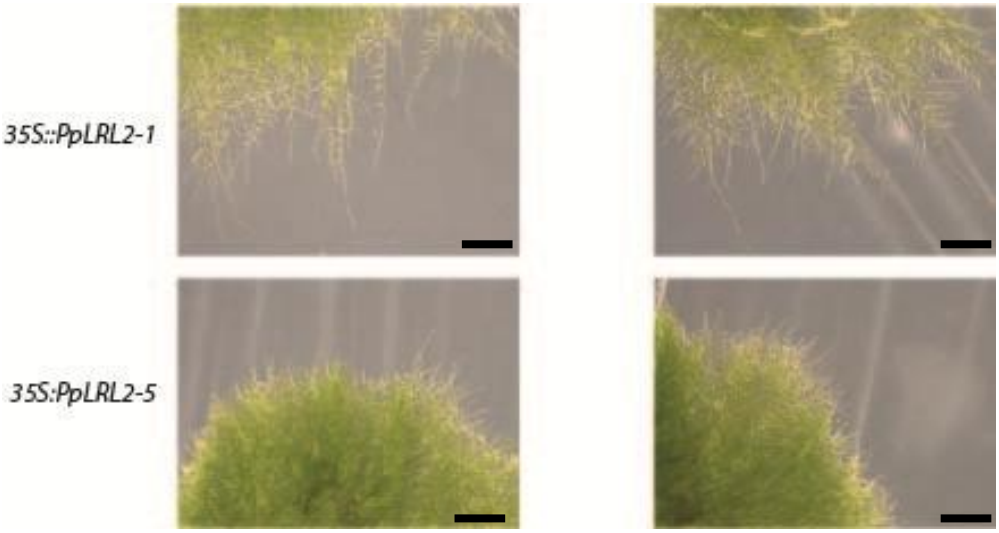


Figure 3.4 – The edges of *Pplrl1* and *Pplrl2* colonies are less caulonema-like than WT colonies

Images of protonema edges of protonema grown from spores germinated on minimal media overlaid with cellophane disk. *Pplrl1* and *Pplrl2* lines (and *35S::PpLRL2-5*) develop few and shorter caulonema-like filaments. Pictures were taken 21 and 28 days after germination. Scale bars indicate 500 µm.

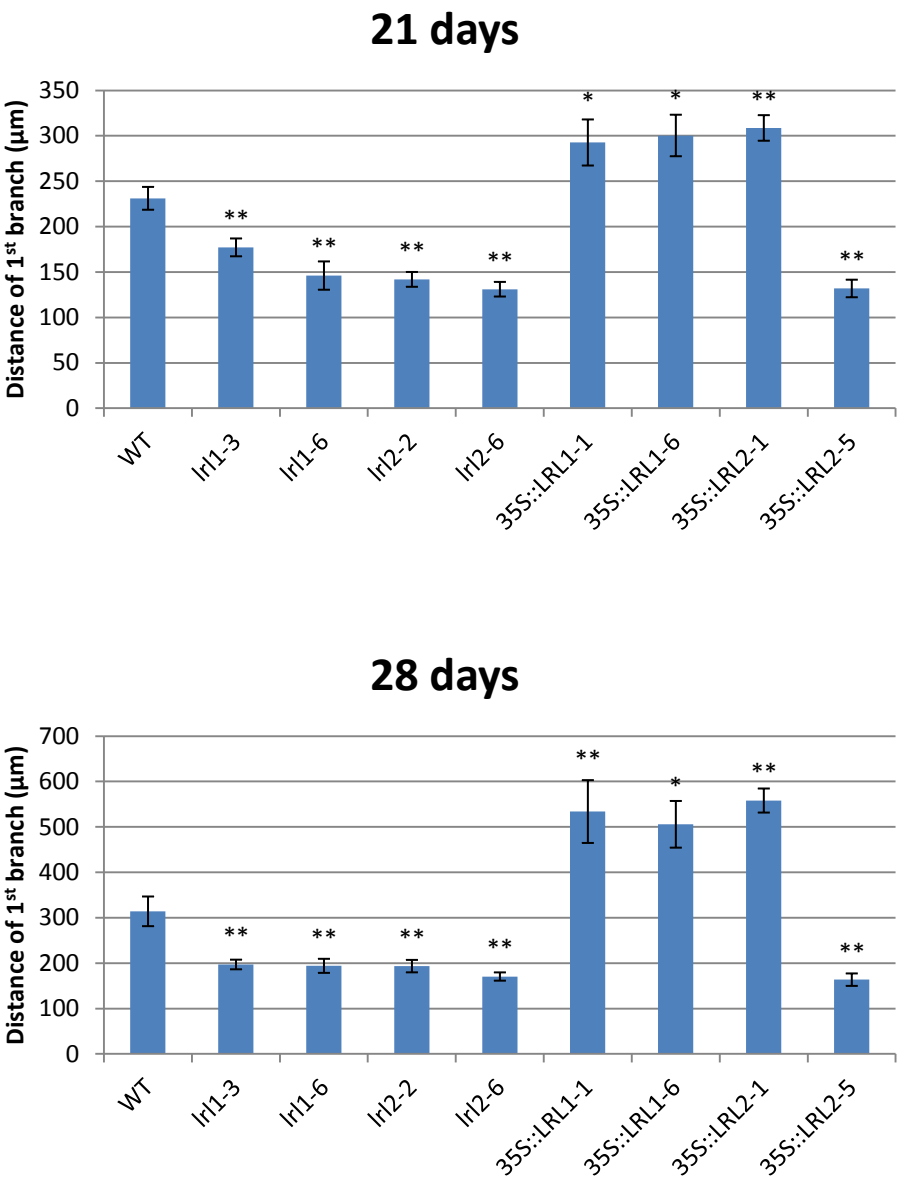


Figure 3.5 – *PpLRL1* and *PpLRL2* are necessary for caulonema development.

Blue bars indicate distance from the tip of a filament to the first side branch; error bars indicate s.e.m., n=15. All loss-of-function mutants and *35S::PpLRL2-5* line develop the first side branch nearer the filament tip while *35S::PpLRL1-1*, *35S::PpLRL1-6* and *35S::PpLRL2-1* develop the first side branch further away from the filament tip compared with WT. Asterisks indicate values that are statistically significantly different from WT ($p<0,05$ and $p<0,01$ (t-test) for single and double asterisks respectively).

PpLRL proteins are required for gametophore development

Protonema colonies growing on minimal media initiate gametophore differentiation 3-4 weeks after spore germination. After 21 days, the number of gametophores is reduced in *Pplrl2-6* compared to wild type, in contrast more gametophores develop in *35S::PpLRL1-1* and *35S::PpLRL1-6* than wild type (Fig. 3.6). The difference of the number of gametophores is greater 28 days after germination between WT and *Pplrl1* and *Pplrl2* lines, where fewer gametophores are formed in all loss-of-function lines. It is observed an increased number of gametophores in *35S::PpLRL1-1* and *35S::PpLRL1-6* compared with the WT plants, while *35S::PpLRL2-5* showed reduced number of gametophores. These results indicate that PpLRL1 and PpLRL2 are positive regulators of gametophore differentiation.

3.1.3. PpLRL proteins are required for rhizoids differentiation

To investigate the role of *PpLRL* genes in rhizoids development, single gametophores were isolated from protonema colonies 31 days after spore germination (Fig. 3.7). All loss-of-function and overexpression lines develop rhizoids; however the pigmentation is defective in all mutant lines. *Pplrl1-3* and *Pplrl1-6* mutant rhizoids have less brown pigmentation than wild type. The defect was stronger in *Pplrl2-2* which develops very pale rhizoids with little pigmentation. No gametophores developed on *Pplrl2-6* colonies. In contrast, rhizoid pigmentation in both *35S::PpLRL1* and *35S::PpLRL2* rhizoids was more intense than in WT.

The phenotypic analysis of rhizoids is not easy because rhizoids grow underneath protonema colonies and their visualization therefore is not possible when protonema colonies grow on plates. To address this issue, I plated WT spores on minimal media overlaid with a cellophane disk for 21 days and then I transferred the protonema colonies to tubes with different media compositions to identify the best condition to visualize rhizoids (Appendix 4). The use of phytagel instead of agar as gelling agent in the media allows a better visualization of rhizoids because media supplemented with phytagel is more transparent than media supplemented with agar. After five days of growth on phytagel it was possible to visualize rhizoids growing into the media without dissection (Appendix 4). *35S::PpLRL1* rhizoids were longer and were more intensely pigmented thatn WT (Fig. 3.8). The differentiation of rhizoids in *35S::PpLRL2-1* is similar to WT. *35S::PpLRL2-5* has slightly shorter rhizoids and less brown-pigmentation compared to WT, consistent with the hypothesis that *PpLRL2* is co-suppressed in this background. All the *Pplrl1* and *Pplrl2* lines also exhibit slightly shorter and less brown-pigmented rhizoids. These results suggest that PpLRL proteins are necessary for

the growth and differentiation of rhizoids, specifically for rhizoids pigmentation. It was not possible to determine the number of rhizoids in these plants.

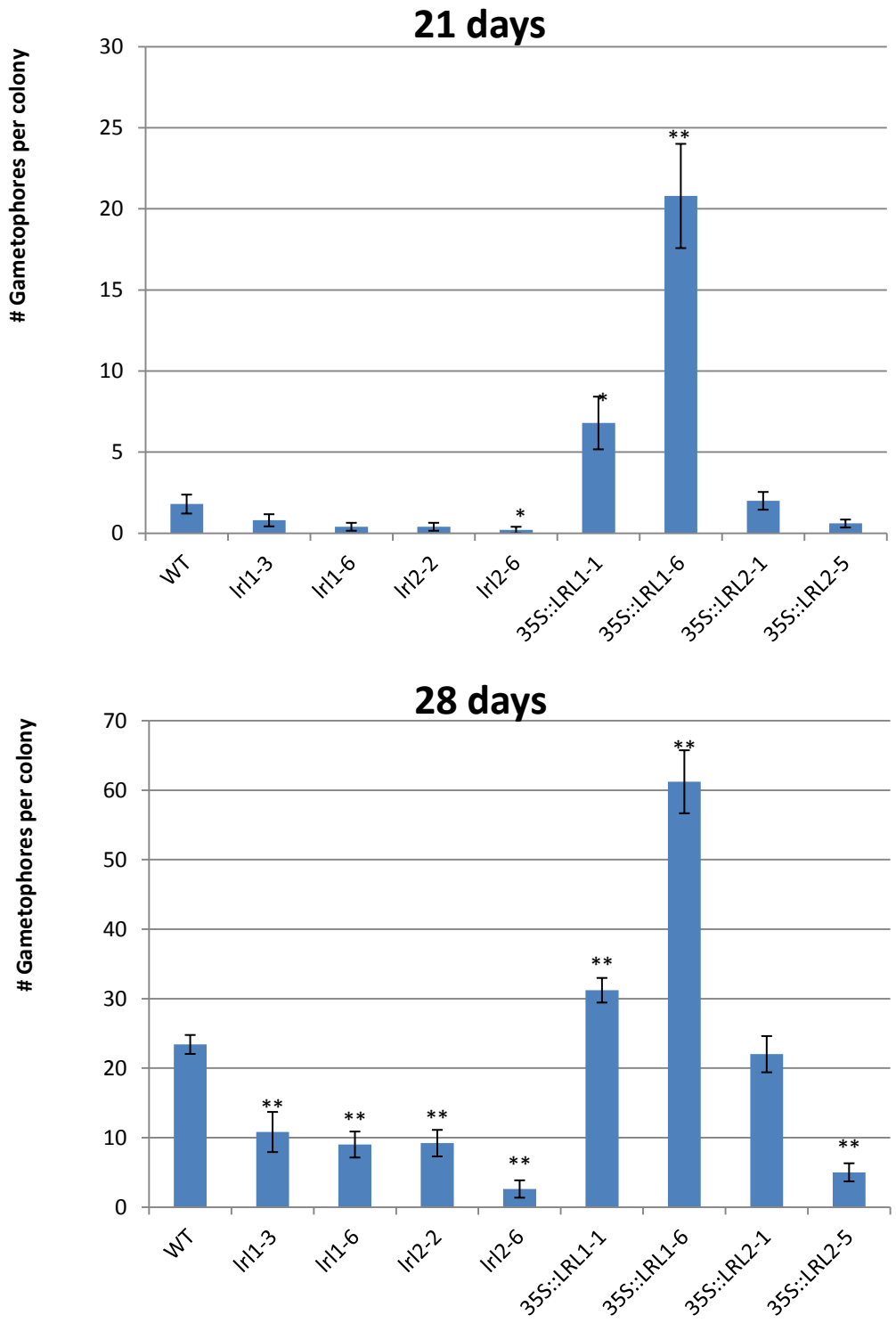


Figure 3.6 – Gametophore development is positively controlled by *PpLRL*

Spores were germinated on minimum media with cellophane disks. Number of gametophores per colony is represented by blue bars and it is higher in both *35S::PpLRL1* lines at 21 days. This increase is also observed at 28 days as well as a decreased number of gametophores in all loss-of-function lines and *35S::PpLRL2-5*. Error bars indicate s.e.m., n=5. Asterisks indicate values that are statistically significant different from WT ($p<0.05$ and $p<0.01$ (t-test) for single and double asterisks respectively).

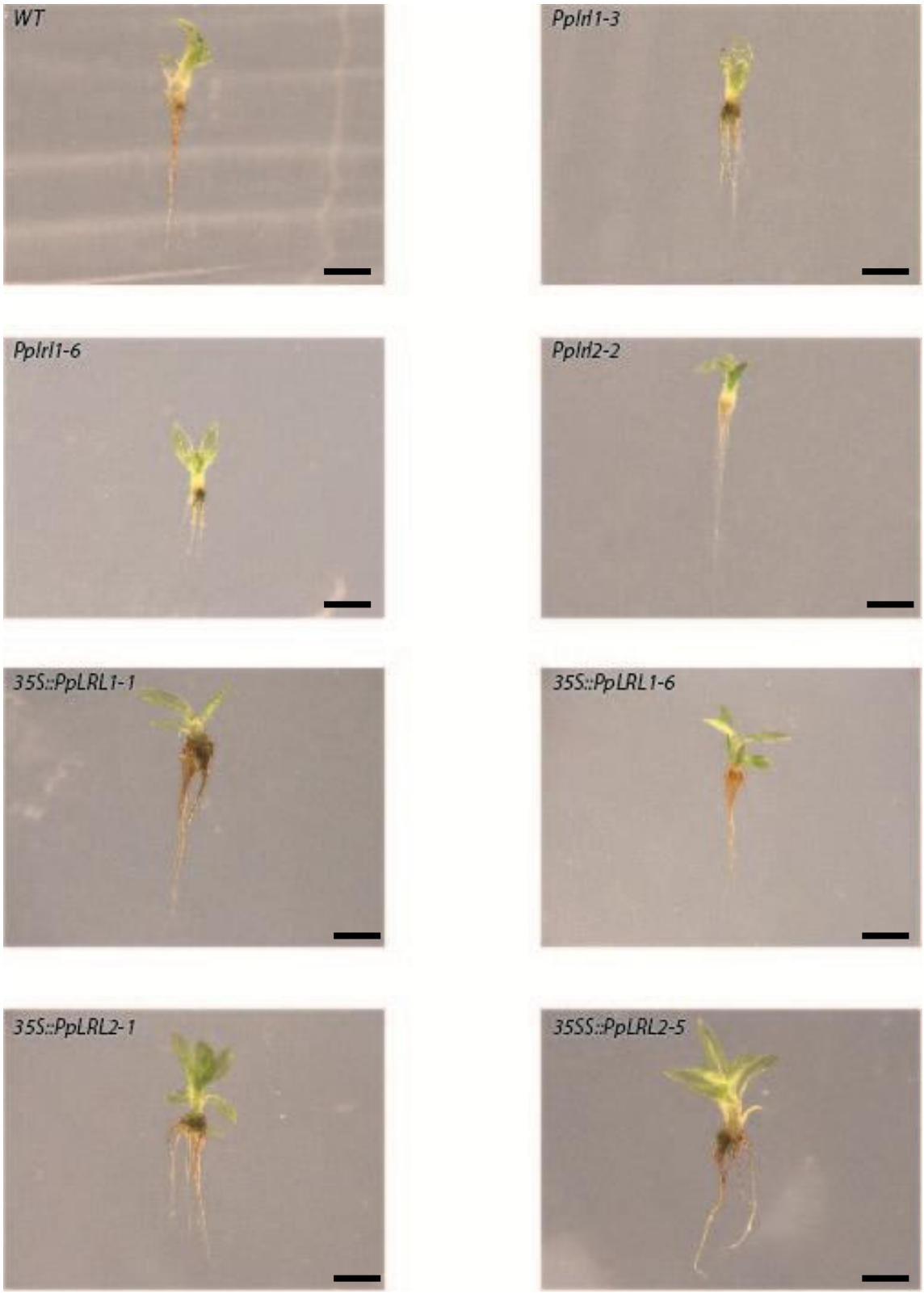
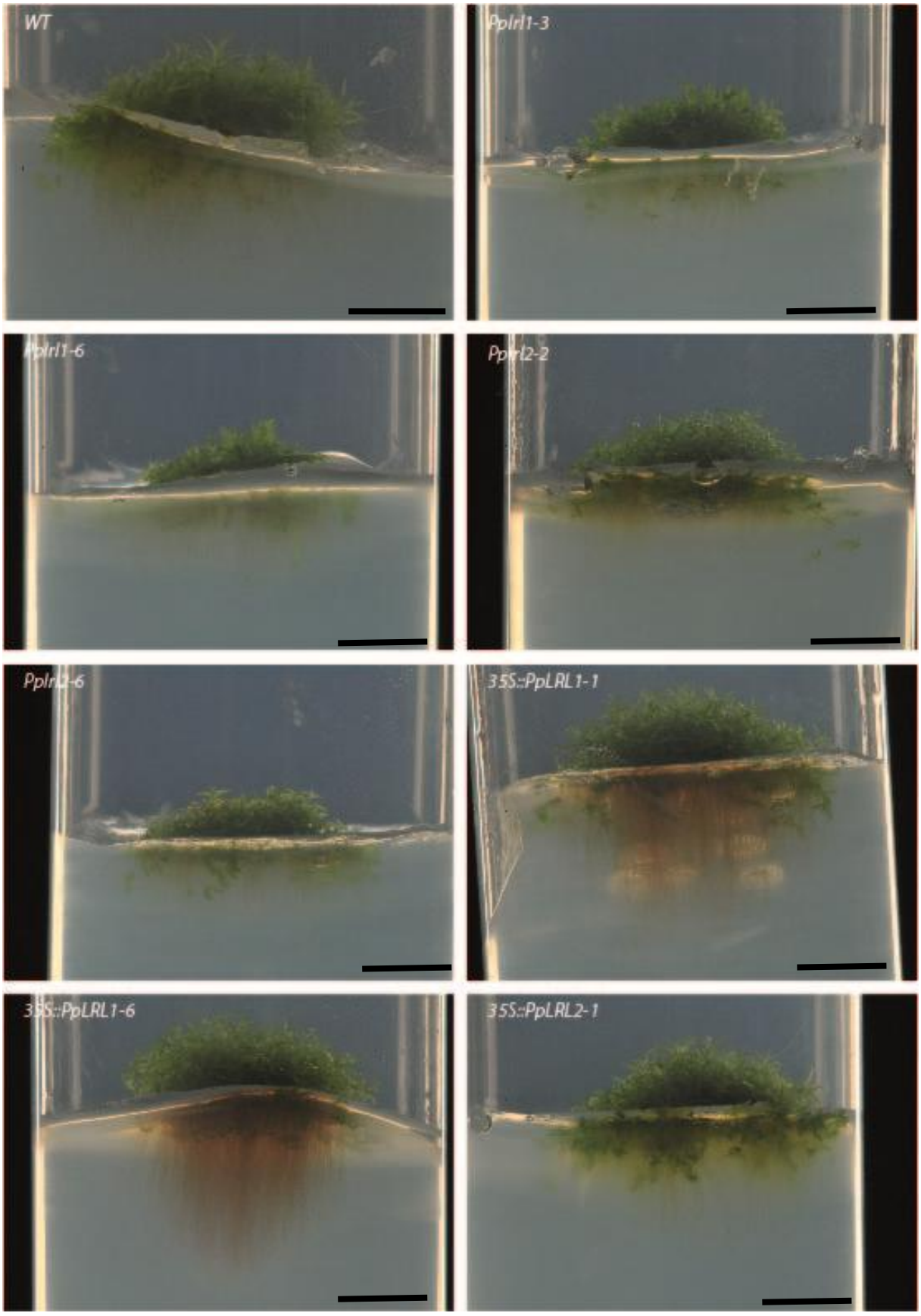


Figure 3.7 – Rhizoid pigmentation is controlled by PpLRL proteins

Gametophores isolated from 31 days old protonema colonies growing in minimal media overlaid with cellophane disk. Brown pigmentation is defective in all loss-of-function lines whereas all overexpressed lines show darker brown-pigmented rhizoids when compared with WT. Scale bars indicate 500 μm.


WT
Pplrl1-3
Pplrl1-6
Pplrl2-2
Pplrl2-6
35S::PpLRL1-1
35S::PpLRL1-6
35S::PpLRL2-1

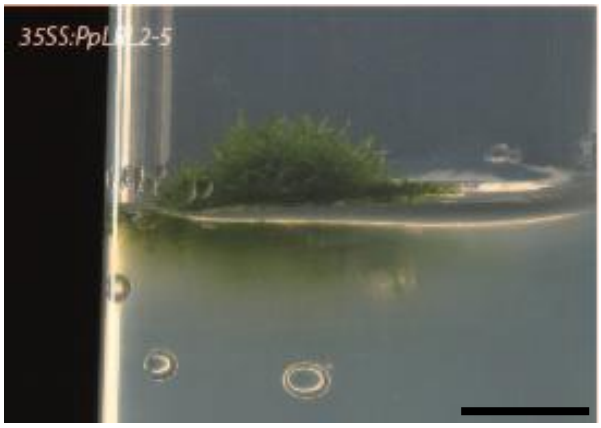


Figure 3.8 – PpLRL proteins positively regulate rhizoids development

Seven-week old protonema colonies growing on minimal media with phytagel. *Pplrl1*, *Pplrl2* and *35S::PpLRL2-5* lines develop shorter rhizoids whereas *35S::PpLRL1-1* and *35S::PpLRL1-6* develop longer and dark brown-pigmented rhizoids. Scale bars indicate 5 mm.

3.1.4. Auxin-induced caulonema and rhizoid differentiation requires *PpLRL1* and *PpLRL2* function

To determine whether auxin controls the development of caulonema and rhizoids by regulating *PpLRL1* and *PpLRL2* function, the sensitivity of *Pplrl1-6* and *Pplrl2-6* mutants to auxin-treatment was determined. Auxin-treatment of WT, *Pplrl1-6* and *Pplrl2-6* lines with 0.1 μM NAA for 23 days resulted in the growth of smaller colonies compared to untreated controls (Fig. 3.9). Exogenous application of 0.1 μM NAA in WT moss resulted in increased development of caulonema compared to untreated controls; every chloronemal cell differentiated into caulonema (Fig. 3.10). By contrast few caulonemal cells developed in auxin-treated *Pplrl1-6* lines, resembling the untreated *Pplrl1-6* control (Fig 3.10). This indicates that the induction of caulonema by auxin requires *PpLRL1* activity. Auxin can induce caulonema development in *Pplrl2-6*. However the auxin-treated *Pplrl2-6* develops significantly less caulonema than the auxin-treated WT, which suggests that the induction of caulonema by auxin only partially requires *PpLRL2* activity (Fig. 3.10). There is an increase in the number of rhizoids, gametophores and rhizoids pigmentation in WT moss treated with 0.1 μM NAA compared with untreated controls (Fig. 3.11). Auxin-treated *Pplrl1-6* also develops more rhizoids on gametophores compared with untreated controls; however these rhizoids had no brown-pigmentation, similar to the rhizoids of the untreated *Pplrl1-6* controls (Fig. 3.11). Auxin-treated *Pplrl2-6* was morphologically identical to WT, although the level of brown-pigmentation was lower in treated *Pplrl2-6* lines compared with treated WT (Fig. 3.11). Together, these results suggest that auxin-induced caulonema and rhizoid differentiation requires *PpLRL1* function and partially requires *PpLRL2* function.

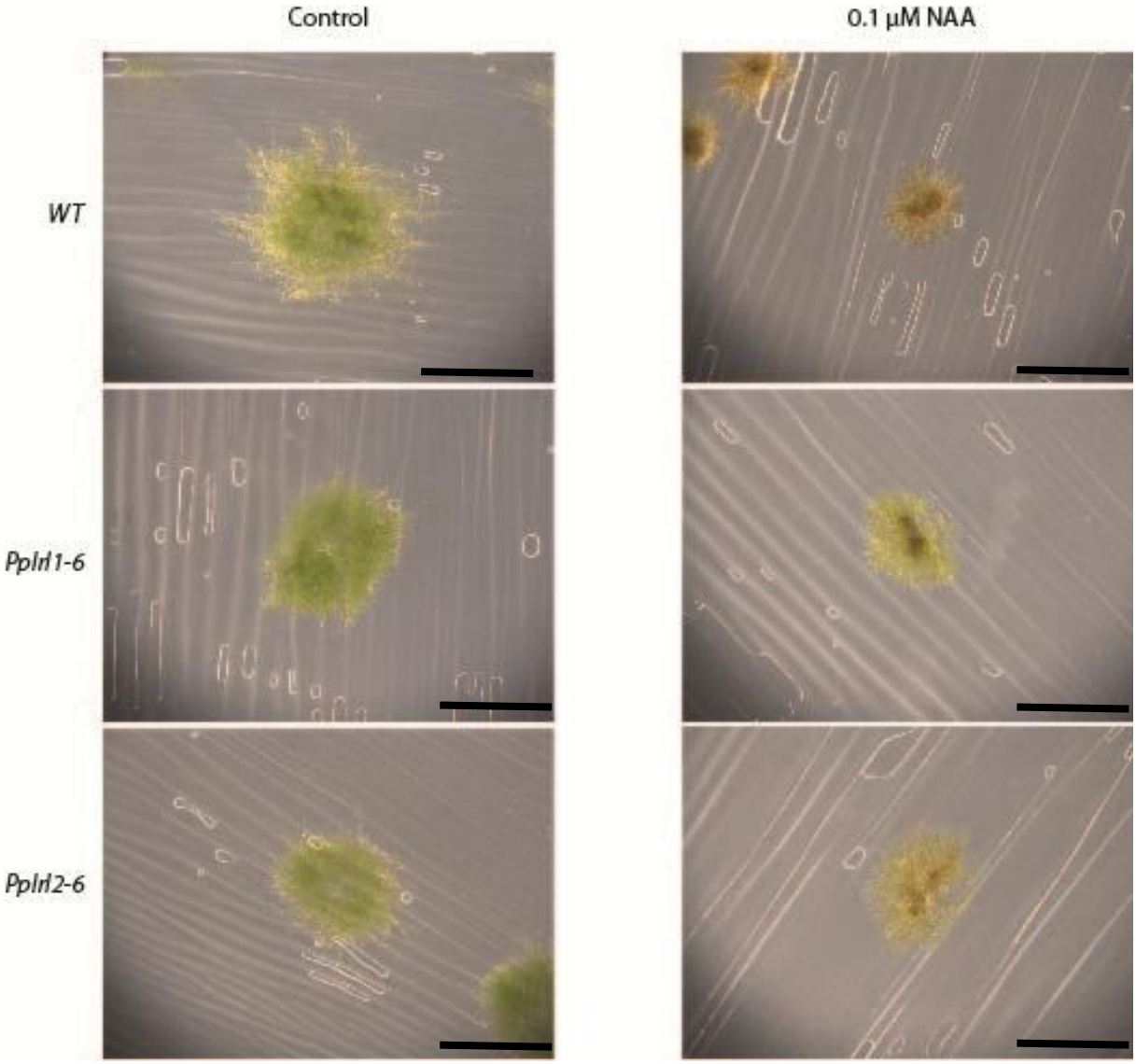


Figure 3.9 – Auxin treatment with 0.1 µM NAA resulted in smaller colonies

Spores were germinated on minimal media (Control) and minimal media supplemented with 0.1 µM NAA (0.1 µM NAA treated). All treated lines show smaller colonies compared with the respective untreated controls Pictures were taken 23 days after germination. Scale bars indicate 5 mm.

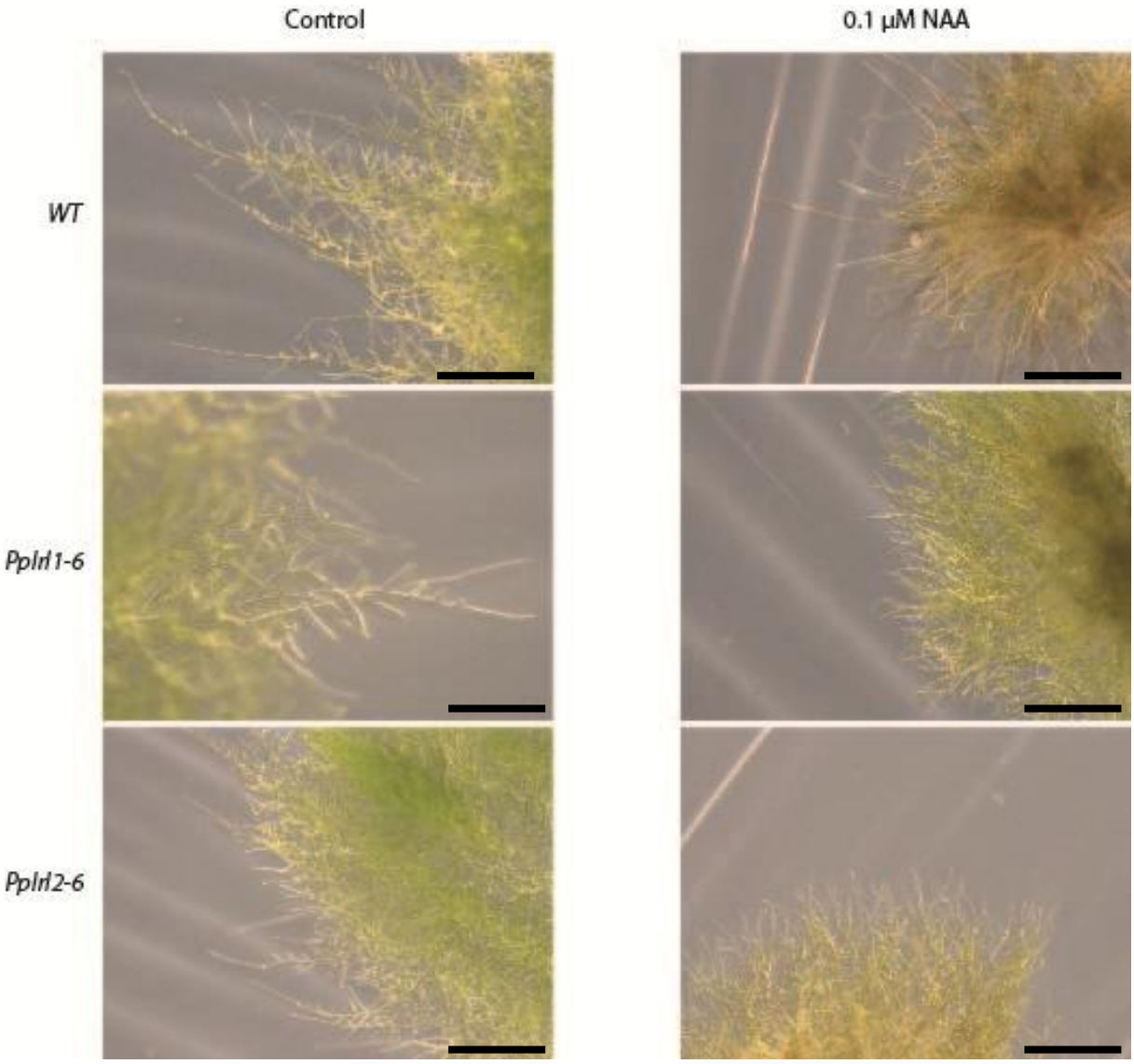


Figure 3.10 – *PpLRL1* gene activity is required for the induction of caulonema differentiation by auxin.

Images of edges of protonemata grown from spores germinated on minimal media (Control) and minimal media supplemented with 0.1 µM NAA (0.1 µM NAA treated). Caulonema differentiation is insensitive to NAA in *Pplrl1-6* and partially sensitive in *Pplrl2-6*. Pictures were taken 23 days after germination. Scale bars indicate 1 mm.

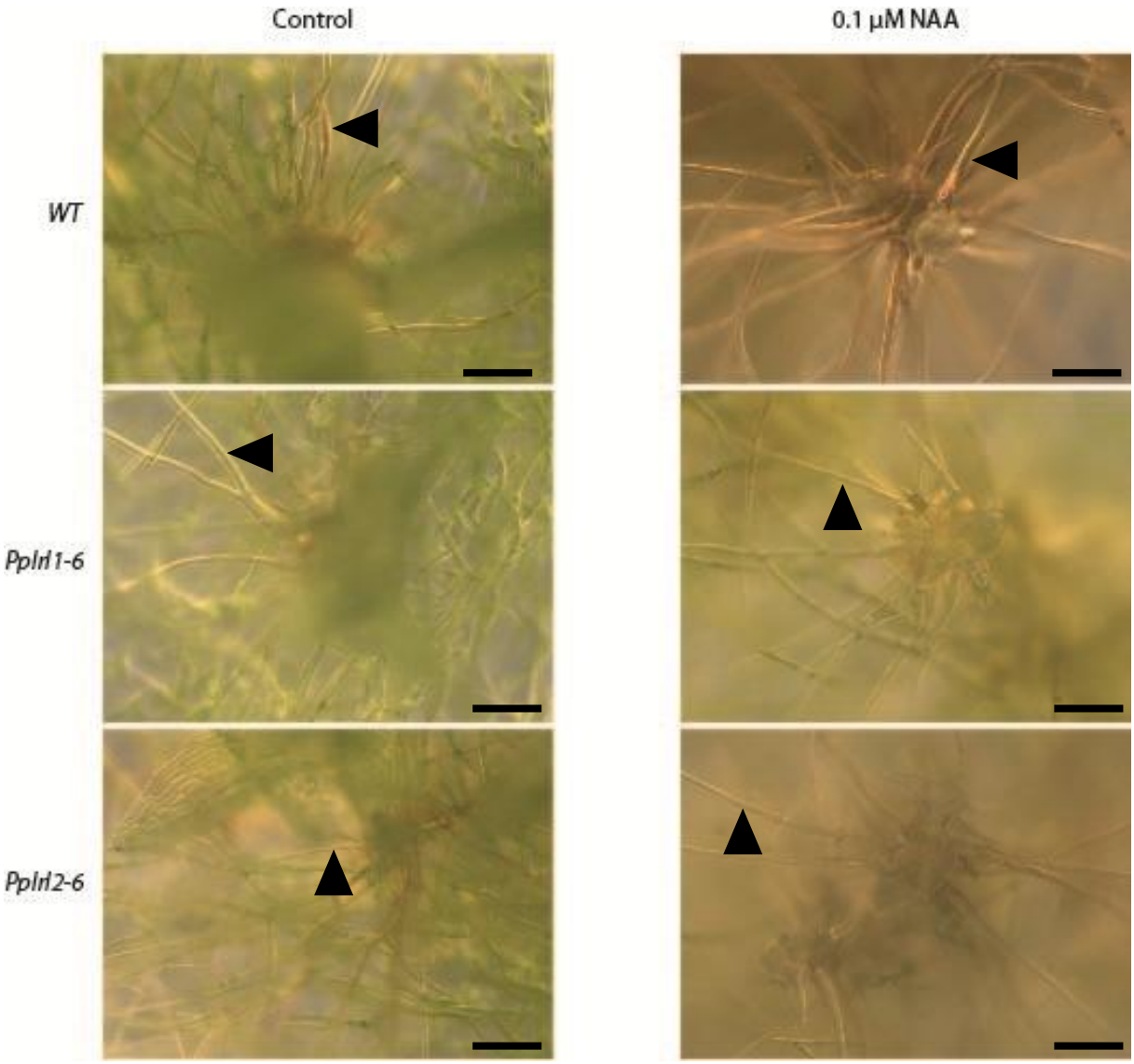


Figure 3.11 – Induction of rhizoid pigmentation requires the activity of *PpLRL1* gene

Images of rhizoids (black arrowheads) growing from a gametophore of colonies growing from spores germinated on minimal media (Control) and minimal media supplemented with 0.1 µM NAA (0.1 µM NAA treated). Rhizoid pigmentation is insensitive to NAA in *Pplrl1-6* and slightly sensitive in *Pplrl2-6*. Pictures were taken 23 days after germination. Scale bars indicate 200 µm.

3.1.5. *Physcomitrella patens LRL* genes are not regulated by RSL Class I

In *A. thaliana*, the transcription of *AtLRL3* gene is positively regulated by RSL Class I proteins (Karas et al., 2009; Bruex et al., 2012). To determine if *RSL* Class I genes control the expression of *PpLRL* genes in *P. patens*, the expression level of *PpLRL* genes in 14 days old *Pprsl1 Pprsl2* protonema grown from macerated protonema was determined by qRT-PCR (Fig. 3.12). *PpLRL1* and *PpLRL2* expression levels in *Pprsl1 Pprsl2* protonema are 0.68x and 0.70x, respectively, of their expression levels in WT, which does not meet the threshold of 0.50x that is normally accepted as a significant reduction. This indicates that *RSL* Class I genes do not regulate *PpLRL* genes in *P. patens* as they do in *A. thaliana* and suggests that other factors promote *PpLRL* expression.

3.1.6. Both *PpLRL1* and *PpLRL2* are negatively regulated by NAA

Auxin is a key player in the root hairs transcriptional network of *A. thaliana*: exogenous NAA does not regulate the expression of *RSL* Class I genes but it positively regulates the expression of *AtRSL4*, *AtRSL5* and *AtLRL3* and negatively regulates *AtRSL2* and *AtRSL3* (Karas et al., 2009; Yi et al., 2009; Bruex et al., 2012). Hairless *Atrhd6 Atrsl1* plants treated with NAA can develop root hairs, meaning that NAA can bypass *RSL* Class I genes and directly activate the transcription of downstream targets (Yi et al., 2010; Bruex et al., 2012). In *P. patens*, auxin positively regulates the differentiation of caulonema and rhizoids by positively regulating the transcription of *RSL* Class I genes (Jang and Dolan, 2011; Jang et al., 2011). Because *PpLRL1* and *PpLRL2* also control caulonema and rhizoid differentiation, and especially since these processes are insensitive to NAA in the *Pplrl1* mutant, it is suggested that auxin may play an important role in the transcriptional regulation of *LRL* genes in *P. patens*.

To test this hypothesis, WT *Physcomitrella patens* spores were plated and protonema grown for three weeks on minimal media supplemented with 0.1 µM NAA . The transcriptional level of *LRL* genes was then determined by qRT-PCR (Fig. 3.13). Surprisingly, the expression of both *PpLRL1* and *PpLRL2* genes is negatively regulated by NAA; the levels of expression of *PpLRL1* and *PpLRL2* in WT treated with 0.1 µM NAA are 0.28x and 0.44x, respectively, compared to WT non-treated, which meets the threshold of 0.50x that is normally accepted as a significant reduction. This result suggests that the regulation of *LRL* expression in *P. patens* and *A. thaliana* is different: IAA positively regulates *AtLRL3* expression in *A. thaliana* while IAA negatively regulates both *PpLRL1* and *PpLRL2* expression in *P. patens*.

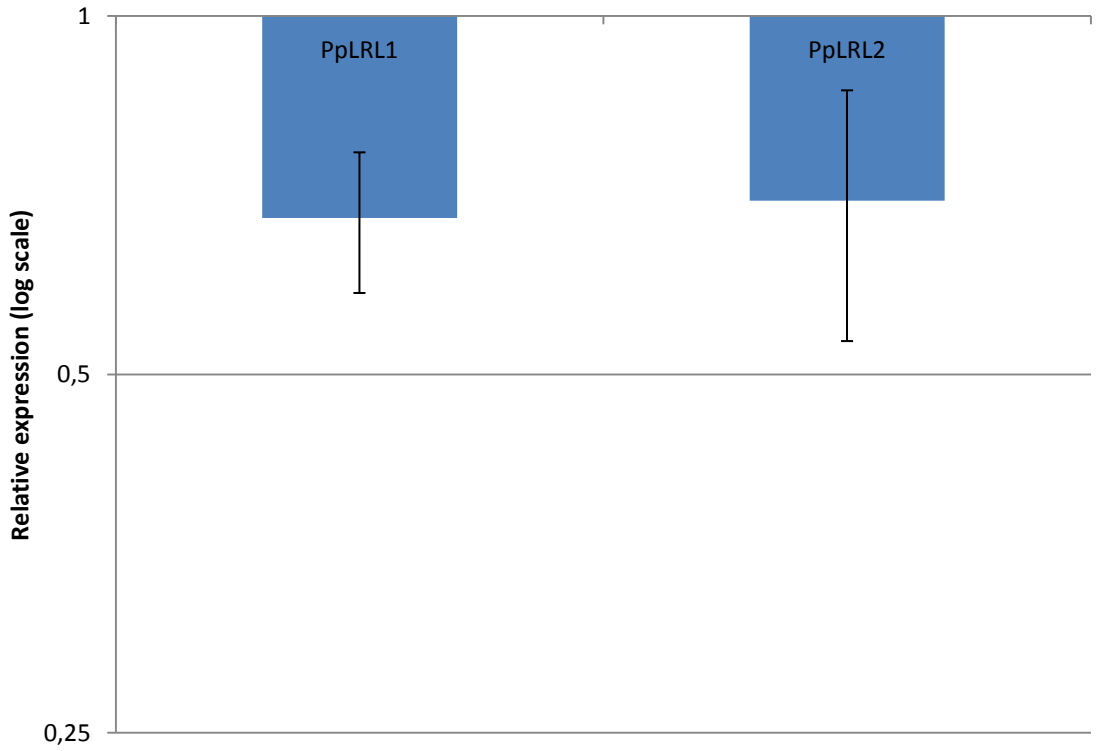


Figure 3.12 – Expression level of *LRL* genes is independent of RSL Class I proteins.

RNA was extracted from 14 days old WT and *Pprsl1* and *Pprsl2* protonema growing on minimal media overlaid with cellophane disk. The expression levels were determined by qRT-PCR, each value corresponds to the expression level relative to WT. Both expression of *PpLRL1* and *PpLRL2* genes are not regulated by RSL Class I proteins. The mean and s.e.m. of three biological replicates are indicated.

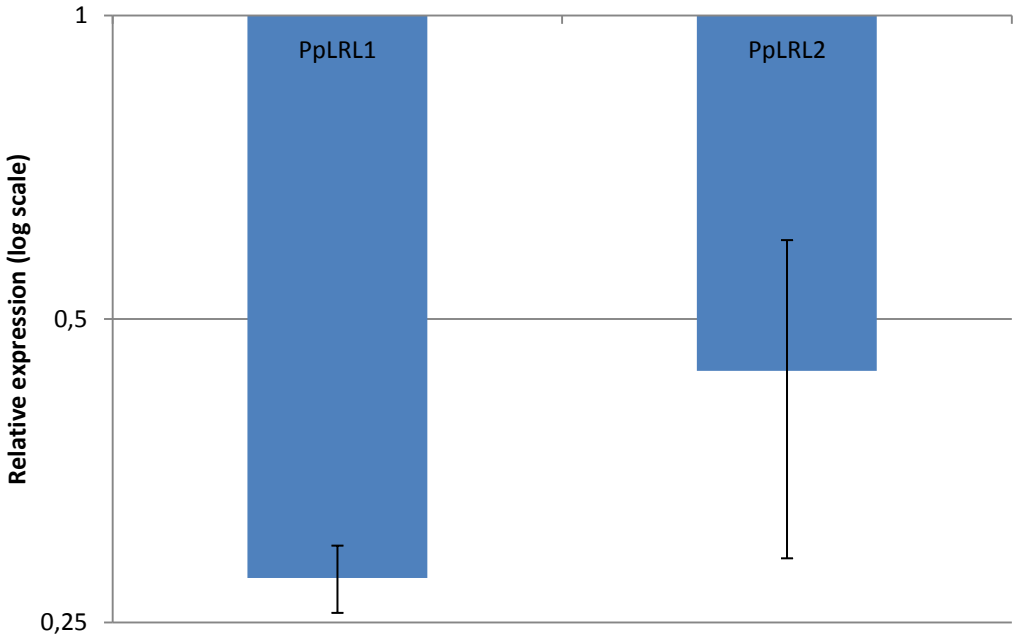


Figure 3.13 – Expression level of *LRL* genes is negatively controlled by NAA

RNA was extracted from 21 days old WT protonema growing on minimal media overlaid with cellophane disk (untreated control) and on minimal media supplemented with 0.1 µM NAA overlaid with cellophane disk (treated). The expression levels were determined by qRT-PCR, each value corresponds to the expression level relative to untreated WT. Both expressions of *PpLRL1* and *PpLRL2* genes are negatively regulated by NAA. The mean and s.e.m. of three biological replicates are indicated.

3.2. Discussion

In *Arabidopsis*, *RSL* Class I and *LRL* genes form a transcriptional network that controls the development of root hairs (Karas et al., 2009; Bruex et al., 2012). A functional characterisation of *RSL* Class I genes showed that they control the development of rhizoid and caulonema filaments in *P. patens* (Menand et al., 2007b). This suggests that a network of *RSL* Class I and *LRL* genes controlled the development of rooting cells in early land plants, and this network has been used to control the development of cells with a rooting function in land plants. The study presented in this chapter supports this hypothesis, and shows that the transcriptional regulatory interactions between RSL and LRL have changed considerably during land plant evolution.

If a developmental mechanism involving *RSL* Class I and *LRL* genes controlled the development of rooting cells in early plants, it is expected that LRL proteins would also control the development of rhizoids and caulonemata in *P. patens*. Supporting this hypothesis, PpLRL1 and PpLRL2 are sister to LRL proteins that control root hair development in *Arabidopsis;* AtLRL1, AtLRL2 and AtLRL3 (Fig. 3.1. The phylogenetic analysis carried out in this study does not allow the inference of the number of *LRL* genes in the common ancestor of *A. thaliana* and *P. patens*. This can be determined by sampling more taxa and including them in the phylogenetic analysis in the future as more bryophyte genome sequences become available.

The phenotypic analysis of plants with altered levels of *PpLRL* gene expression demonstrates that both *PpLRL1* and *PpLRL2* are required for the development of rooting cells in moss. Loss-of-function *Pplrl1* and *Pplrl2* develop fewer caulonema cells while *35S::PpLRL1* and *35S::PpLRL2* overexpressed lines develop more caulonema-like filaments than wild type (Figs 3.3, 3.4 and 3.5). Both loss-of-function mutants develop rhizoids, but these were defective in their pigmentation (less brown-pigmentation), whereas in both overexpressed mutants they developed longer rhizoids and they showed more brown-pigmentation (Figs. 3.7 and 3.8), which indicates that both *PpLRL1* and *PpLRL2* are required for rhizoids differentiation. All of the independent loss-of-function mutans and overexpression lines show concordant results with one exception. *35::PpLRL2-5* always shows a phenotype comparable with the loss-of-function *Pplrl2* phenotype which indicates that the gene might be silenced in this line. This phenomenon is called “co-suppression” and is common in plants, where overexpression of a gene may lead to decrease levels of its expression by RNAi (Meyer, 1998). Analyse of gene expression by qRT-PCR is needed to validate which overexpressed lines are really overexpressing the gene.

Data presented here indicate that *LRL* genes are not only required for rhizoid and caulonema development. Gametophore development also requires *PpLRL* activity. *Pplrl1* and *Pplrl2* produce fewer gametophores than wild type, while more gametophores develop in plants that overexpress *PpLRL1* (Fig. 3.6). Similarly *AtLRL* genes in *A. thaliana* are involved in root hair development and other developmental processes. For example while *AtLRL1* and *AtLRL2* mutants are required for root hairs, the *Atlrl1 Atlrl2* loss-of-function mutant is lethal in *A. thaliana*, indicating that these genes also function in other developmental processes apart from root hair development (Karas et al., 2009). A more detailed study of LRL function in *A. thaliana* is necessary to test this hypothesis. It is possible that *LRL* genes were recruited from the gametophyte generation to the sporophyte generation during land plant evolution to control not only the development of cells with rooting function but also other developmental processes.

It would be instructive to determine the phenotype of the double *Pplrl1 Pplrl2* mutant to test if *PpLRL1* and *PpLRL2* act in a partially redundant way to control the development of cells with a rooting function. Because both *LRL* genes are required for caulonemata and rhizoids development, I hypothesise that the double mutant would have a more severe phenotype, similar to the double *Pprsl1 Pprsl2* mutant, where complete disruption of caulonemata and rhizoid development is observed. Similarly, it would also be instructive to determine the phenotype of a double *PpLRL1* and *PpLRL2* overexpression line to find out whether *LRL* genes are sufficient for rhizoids and caulonemata development or for other developmental process in moss, such as gametophore development.

In *A. thaliana*, the expression of *AtLRL3* is positively regulated by RSL Class I proteins during root hair development (Karas et al., 2009). However, this transcriptional regulation does not occur in *P. patens* where the expression of both *PpLRL1* and *PpLRL2* is independent of PpRSL Class I proteins (Fig. 3.12). It is known that auxin is a key regulator of the differentiation of root hairs in higher plants and both caulonema and rhizoid development in mosses. The topology of the network that comprises auxin, *RSL* Class I and *LRL* genes is very different. For example, auxin is a positive regulator of *RSL* Class I expression in *P. patens* but not in *A. thaliana* (Jang et al., 2011). As expected auxin controls the expressionn of *PpLRL1* and *PpLRL2* genes, but that auxin negatively regulates the expression of these genes was unexpected (Fig. 3.13). Furthermore *PpLRL1* and *PpLRL2* expression is required for rhizoid and caulonema development (Figs. 3.9, 3.10 and 3.11). Together, these facts suggest that regulatory interactions within the auxin-*RSL* Class I-*LRL* gene regulatory network are different in *A. thaliana* and *P. patens;* auxin. *RSL* Class I and *LRL* genes are components of an incoherent network (a network where a single input activates one intermediate pathway and inhibits another intermediate pathway, both of which modulate a

single output) that controls the development of cells with a rooting function in *P. patens* (Fig. 3.14) (Kim et al., 2008). *LRL* may function as a positive modulator of the auxin induction of *RSL* Class I to control rhizoid and caulonema development. With high levels of auxin (resulting in more caulonema and rhizoids), *LRL* expression is lower whereas *RSL* expression is higher, but this decrease of *LRL* expression is not sufficient to inhibit completely the positive regulation of the auxin mediated rhizoids and caulonema development through *RSL* Class I. If there is no *LRL* function, the positive regulation of the auxin mediated rhizoids and caulonema development through *RSL* Class I is absent, resulting in defective rhizoids and caulonema cells development. In contrast, if the level of *LRL* expression is high, the positive regulation of the auxin mediated rooting cells development through *RSL* Class I is enhanced, resulting in more caulonema and longer rhizoids. To support this model, loss-of-function *Ppshi1* and *Ppshi2* mutants (auxin biosynthesis defective mutants, with lower levels of endogenous auxin) exhibit longer and more brown-pigmented rhizoids (Eklund et al., 2010), which could be a result of higher levels of *PpLRL1* and *PpLRL2* and therefore an enhanced auxin induction of rooting cells through *RSL* Class I. However, it is not known whether *LRL* genes modulate: 1) auxin maxima by regulating the expression of auxin biosynthesis or signalling genes; 2) auxin regulation of *RSL* Class I genes; 3) *RSL* Class I transcription; or 4) genes downstream of *RSL* Class I involved in rooting cells development. To address this question, gene expression analysis of *RSL* Class I, auxin biosynthesis and signalling genes in *Pplrl1* and *Pplrl2* background and in auxin-treated *Pplrl1* and *Pplrl2* mutants must be carried out. It is known that bHLH proteins can form heterodimers with other bHLH proteins or another transcription factor to control distinct developmental processes (Murre et al., 1989; Molkentin et al., 1995; Jones, 2004). If this is the case, then apart from the transcriptional interactions, protein-protein interactions may occur between LRL and RSL proteins to form functional heterodimers to control the transcription of a set of genes responsible for rooting cells development. A protein-protein interaction assay with LRL and RSL proteins could reveal whether this is might occur or not. These two different experimental approaches will give us a better insight into the LRL function in the moss *P. patens*.

The results illustrated in this chapter suggest that an ancient developmental mechanism involving auxin, *RSL* Class I and *LRL* genes controlled the development of rooting structures in the first land plants. During land plant evolution, the recruitment of this regulatory network from the gametophyte generation to the sporophyte generation, where it controls the development of root hairs, might have contributed to the increase of size and complexity of the sporophyte generation. Different regulations within this developmental

network may have led to the different morphologies found in the rooting systems in land plants.

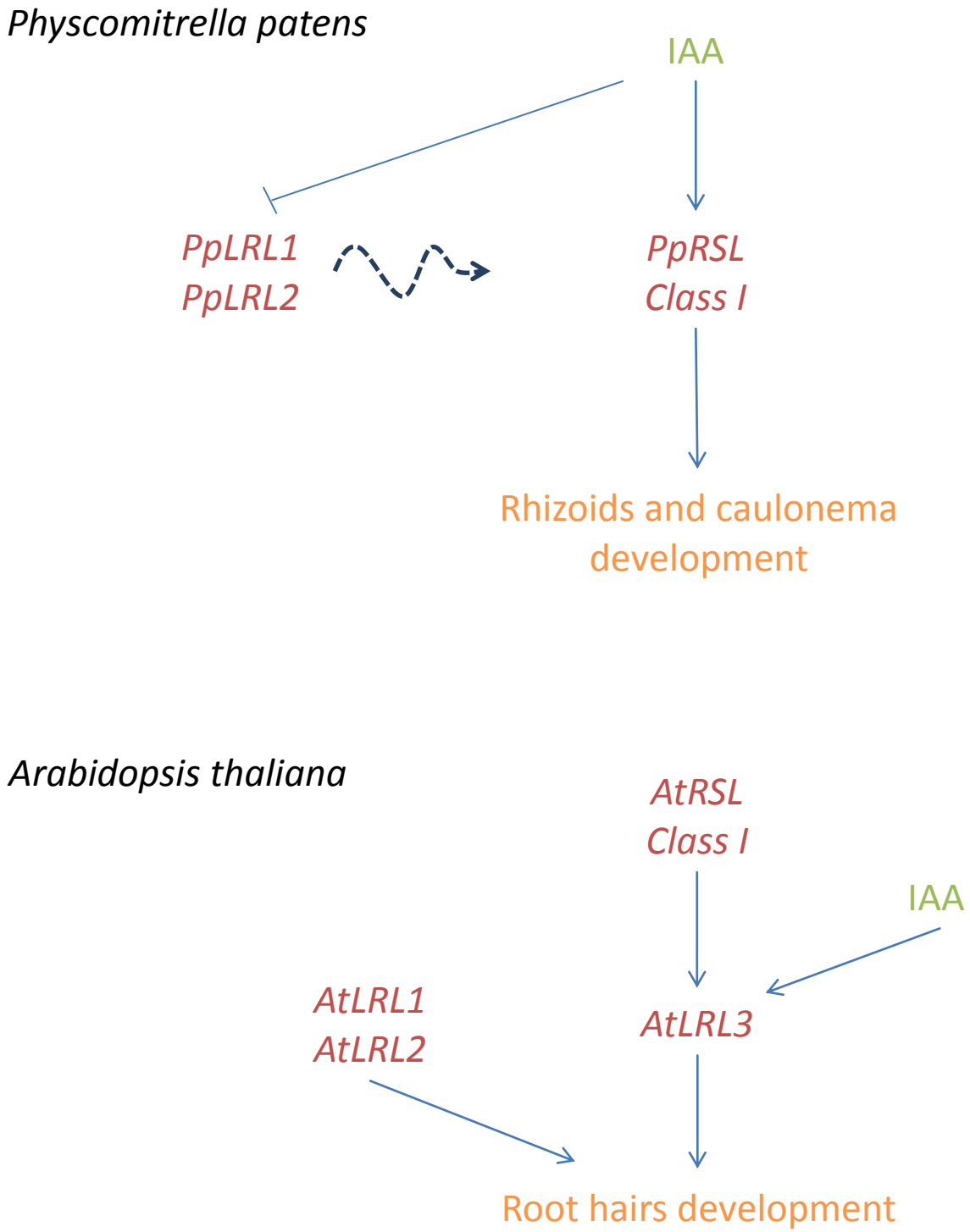


Figure 3.14 – Working model of the rooting cell developmental network in *Physcomitrella* and in *Arabidopsis*.

The transcriptional interactions between IAA (auxin), *RSL* Class I and *LRL* genes are indicated. [→]: positive regulations; [-|]: negative regulations; dashed curved arrow: hypothetical positive regulation of IAA, *RSL* Class I or genes downstream of *RSL* Class I (or the interaction among these components).

Chapter 4. Generation of *Pphol* knock-out line

Methyl halides are volatile compounds derived from methane and play a major role in the destruction of the ozone layer in the stratosphere (Penkett et al., 1980; Schauffler et al., 1998). Plants produce and emit methyl halides and their synthesis is catalysed by methyltransferase protein encoded by *Harmless to Ozone Layer* (*HOL)* in *Arabidopsis thaliana* (Rhew et al., 2003). *Physcomitrella patens* is also able to produce and emit methyl halides (Lars Østergaard, personal communication, August 3, 2012), but whether their synthesis is catalysed by the HOL protein is still unknown. The genome of *P. patens* contains one *HOL* gene but its function has not been yet characterized. Furthermore, the biological function of methyl halides in moss is unknown and their biological function could be studied by generating mutant plants in which the production of methyl halides is defective. We hypothesise that *PpHOL* is required for methyl production in mosses and that this mechanism is conserved among land plants. The generation of a knock-out *Pphol* mutant described in this chapter is intended to test the role of *PpHOL* in methyl halides production and also to study the biological function of methyl halides in moss. This *Pphol* knock-out mutant can then be used to know whether the methyl halides production mechanism is conserved during land plant evolution.

The work presented in this chapter is part of a collaboration with Lars Østergaard lab (John Innes Centre, UK) and the plasmid used for the generation of the *Pphol* knock-out line was produced and provided by Evelyn Koerner (John Innes Centre, UK). Here I report the generation of a *Pphol* knock-out mutant.

4.1. Results

4.1.1. Strategy and generation of mutants

Gene targeting can be easily performed in *P. patens* due to its high rates of homologous recombination (Schaefer and Zrÿd, 1997; Hiwatashi et al., 2001; Hobe et al., 2004; Kamisugi et al., 2005). A genomic locus can be specifically altered by homologous recombination, which allows the generation of complete gene knock-outs, in which an entire target gene can be replaced with an antibiotic resistance gene. The transformation of *P. patens* is usually done by introducing linear molecules of DNA into moss protoplasts using polyethylene glycol (PEG).

In order to generate a *Pphol* knock-out mutant, a fragment upstream of the start codon and a fragment upstream of the stop codon of the *PpHOL* coding sequence (CDS) were cloned into the plasmid pBHrev to make it possible for homologous recombination to occur at the *PpHOL* locus (Fig. 4.1).

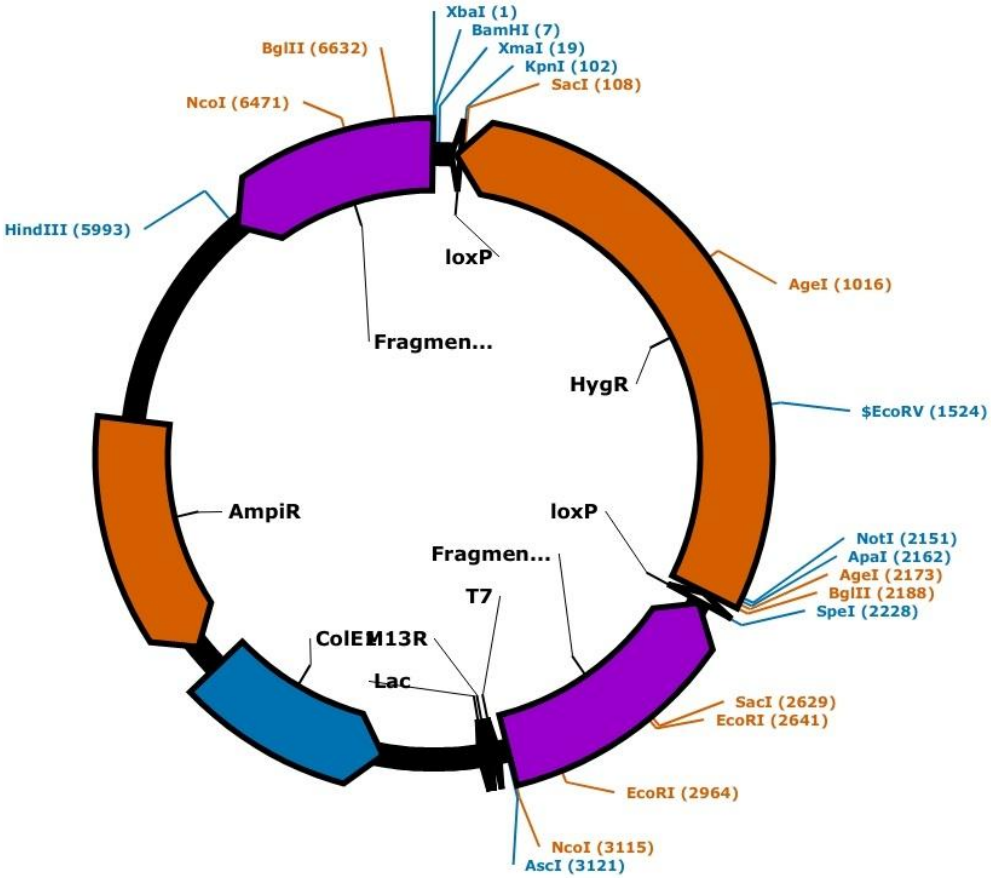


Figure 4.1 – Schematic representation of *PpHOL* knock-out construct.

The hygromycin resistant gene is flanked by the 5' downstream fragment and 3' upstream fragment from the *PpHOL* CDS (represented in purple). Enzymes with multiple restriction sites are represented in orange and enzymes with single restriction site are represented in blue.

The plasmid was double digested with *AscI* and *HindIII* to obtain a linearized DNA fragment containing the hygromycin resistance gene flanked by the upstream and downstream fragments of the *PpHOL* CDS. This is crucial to increase transformation efficiency because when transformation was performed with non-linearized plasmids, the number of transformants was reduced compared with the number of transformants obtained using linearized plasmid.

4.1.2. *Physcomitrella patens* transformation

After plasmid linearization, linear DNA molecules were introduced into *P. patens* protoplasts, obtained by treating five days old macerated protonema with driselase (a cell-wall-digesting enzyme preparation), using PEG. Protoplasts were left to regenerate for six days without selection, and were then transferred to selection plates, which contained hygromycin B (25 μL/mL). After 10 days on the selection plates, resistant transformants were placed into non-selective plates for 10 days to allow plants containing the DNA (transgenic construct), but not having it integrated into the genome, to lose the DNA. A second round of selection was carried out for 10 days and surviving protonema was screened by PCR to verify proper integration at the *PpHOL* locus (Fig 4.2). For PCR, amplification with primers p1/p2 showed integration at the 5' end (amplification in the mutant and no amplification in the WT), and the integration at the 3' end was confirmed with the amplification with primers p3/p4 (amplification in the mutant and no amplification in the WT) (Fig. 4.2). Amplification with primers p5/p6 was done to completely confirm that the gene was knocked out in the mutant lines (amplification in WT and no amplification in the mutant) (Fig. 4.2). Over 200 transformants were recovered after second selection and 20% had the proper homologous recombination integration confirmed by PCR. For the sake of simplicity, the PCR screening for only 5 transformant lines is shown (Fig. 4.3). These mutant lines are ready to be tested for methyl halide emission, although further confirmation by Southern blot analysis for single insertion into the genome is necessary.

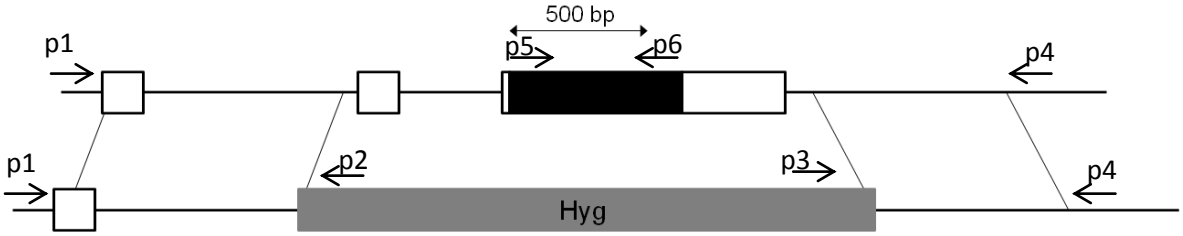


Figure 4.2 – Generation of *hol* knock-out in *P. patens*.

Structure of the *PpHOL* gene (Pp1s304_15V6.2, Phypa_60954) (up) and the expected result in the *Pphol* knock-out after homologous recombination (down). The boxes represent exons with non-coding (white) and coding (black) regions. The grey box indicates the hygromycin resistance gene cassette. The regions of homology used for the gene replacement are delimited by grey lines. Arrows indicate the location of primers (p1-p6) used in the PCRs to confirm the homologous recombination events.

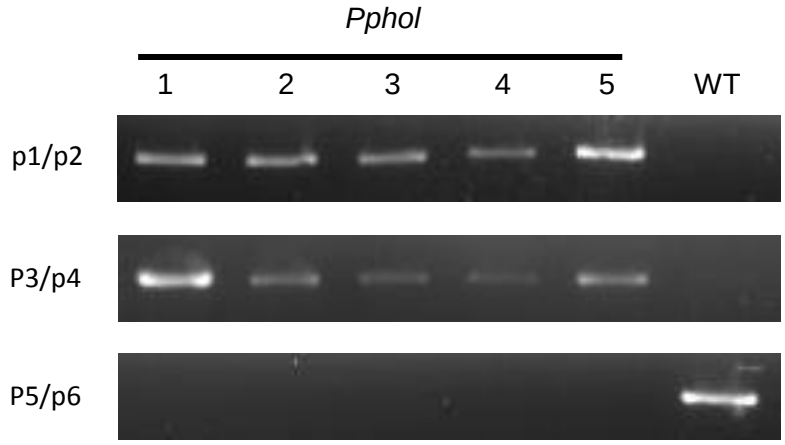


Figure 4.3 – Genotyping of the *PpHOL* locus reveals five independent lines with successful gene targeting by homologous recombination.

Recombination events are confirmed by PCR. Five independent mutant lines have the desired fragment integrated into the *PpHOL* locus, resulting in the replacement of the *PpHOL* gene. The *PpHOL* gene cannot be detected in any of the five independent lines.

4.2. Discussion

Gene targeting is a powerful tool to generate gene knock-outs in *P*. *patens*. The transformation described in this chapter has resulted in the generation of a mutant that will be critical for understanding the evolution of physiological processes in land plants. The loss-of-function *Pphol* mutant could reveal whether a conserved mechanism for the production of methyl halides is present in land plants. This can be tested by quantifying the concentration of methyl halides emitted by the *Pphol* mutant; if methyl halides emission is reduced in the *Pphol* mutant compared to the WT, it demonstrates that *PpHOL* is necessary for the production of methyl halides in moss and that the mechanism of methyl halide production is conserved since the last common ancestor of *P. patens* and *A. thaliana*.

It is not clear why plants produce and emit methyl halides. Apart from the evolutionary insight which can be achieved with the analysis of this mutant, the phenotypic analysis of the *Pphol* mutant might elucidate the biological function of methyl halides in mosses. The HOL protein could be essential for important physiological processes in the first land plants and during land plant evolution its function became redundant but the mechanism of methyl halides production was kept in spite of being not significant for the plant. This scenario can only be validated by the phenotypic analysis of the loss-of-function *Pphol* mutant, the generation of which was described in this chapter.

It is also necessary to verify any event of illegitimate recombination in the mutants where homologous recombination was confirmed, i.e. to avoid any lines with undesirable (i.e. not in the *PpHOL* locus) integration of the fragment in the genome. This can be done by Southern blot analysis which allows the number of transgene copies integrated in the genome to be determined.

Chapter 5.
Conclusion

The conquest of land by plants had impacts on major chemical cycles and the climate of the Earth, but also involved changes in plant developmental and physiological mechanisms. The challenges of the terrestrial environment drove the evolution of a panoply of developmental and physiological adaptations which made possible the successful radiation of land plants. In this thesis I presented the study of a set of genes, *PpLRL*, that controls the development of caulonema and rhizoids in *P. patens*. Loss-of-function *Pplrl1* and *Pplrl2* mutants showed defective caulonema and rhizoid development, whereas *35S::PpLRL1* and *35S::PpLRL2* overexpression lines developed more caulonema and longer rhizoids with more brown pigmentation. These results indicate that *PpLRL1* and *PpLRL2* are necessary for caulonema and rhizoid development.

It is hypothesized that changes in the *cis*-regulatory elements of genes, and consequently, the re-wiring of the gene network that controls rhizoids development, were crucial for the development of root hairs on the sporophyte (Peter and Davidson, 2011; Jones and Dolan, 2012). Here I showed that the genetic network that controls the development of rhizoids in *P*. *patens* and that of root hairs in *A. thaliana* is conserved, but the regulation of this network is different. While in *A*. *thaliana AtLRL3* is positively regulated by RSL Class I and auxin induces the expression of *At*LRL3, in *P. patens* both *PpLRL* genes are independent of RSL Class I and their expression is negatively regulated by auxin. These different topologies of the same network verified in *A*. *thaliana* and in *P. patens* suggest that changes in the regulation of the components of this conserved network are at least partly responsible for the differences observed in land plant rooting systems.

LRL genes also control other developmental processes in *A. thaliana* and in *P. patens*. A detailed study of gene networks that control these developmental processes can give a better insight about the function of LRL proteins in plants. Given that *PpLRL* genes are required for gametophore development it will be interesting to understand the genetic mechanism that underlies this development process and compare it with the genetic mechanism that controls the development of cells with a rooting function. It is possible that a common mechanism involving *PpLRL* genes is responsible for the regulation of different developmental processes in plants.

The generation of knock-out mutants using gene targeting by homologous recombination is very common in *P*. *patens*. In this thesis I described the generation of a *Pphol* knock-out mutant that may be very useful for the understanding of methyl halide production in *P. patens*. It will demonstrate whether a possible physiological mechanism is conserved among land plants.

These two different approaches, characterization of a gene network and a physiological process in *P*. *patens*, can give us important clues about the mechanisms that occurred during land plant evolution which made it possible for plants to live in a terrestrial environment. The study reported in this thesis also showed that the genes responsible for the development of root hairs in higher plants are also present in basal plants, where they regulate the development of rhizoid. This is very important because it suggests that in the first land plants a set of genes to control rhizoid development were already present, and this set of genes were recruited to the sporophyte generation, where they control the development of root hairs, contributing to the increase of size and complexity of the sporophyte.

References

Abascal, F., Zardova, R. and Posada, D. (2005). ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* **1,** 2104-2105.

Abramoff, M.D., Magalhaes., P.J. and Eliceiri, K.W. (2004). Image Processing with ImageJ. *Biophotonics International* **11,** 36-42.

Alicke, B., Hebestreit, K., Stutz, J. and Platt, U. (1999). Iodine oxide in the marine boundary layer. *Nature* **397,** 572-573.

Ashton, N.W., Cove, D. and Featherstone, D.R. (1979). The isolation and physiological analysis of mutants of the moss Physcomitrella patens. Planta**144**: 437–442. (1979).

Ashton, N.W., Grimsley, N.H. and Cove, D.J. (1979). Analysis of gametophytic development in the moss, *Physcomitrella patens*, using auxin and cytokinin resistant mutants. *Planta* **144,** 427-435.

Aoyama, T., Hiwatashi, Y., Shigyo, M., Kofuji, R., Kubo, M., Ito, M. and Hasebe, M. (2012). AP2-type transcription factors determine stem cell identity in the moss *Physcomitrella patens*. *Development* **139,** 3120-3129.

Berner, R.A. (2001). The effect of the rise of land plants on atmospheric CO_2 during the Paleozoic. pp. 173-178. *In:* P.G. Gensel and D. Edwards (eds.). *Plants Invade the Land: Evolutionary and Environmental Approaches.* New York: Columbia Univ. Press.

Bernhardt, C., Lee, M.M., Gonzalez, A., Zhang, F., Lloyd, A. and Schiefelbein, J. (2003). The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the *Arabidopsis* root. *Development* **130,** 6431-6439.

Bierfreund, N.M., Reski, R. and Decker, E.L. (2003). Use of an inducible reporter gene system for the analysis of auxin distribution in the moss *Physcomitrella patens*. *Plant Cell Rep* **21,** 1143-1152.

Booker, J., Chatfield, S. and Leyser, O. (2003). Auxin acts in xylem-associated or medullary cells to mediate apical dominance. *Plant Cell* **15,** 495-507.

Bopp, M. (1980). The hormonal regulation of morphogenesis in mosses. *– In Proceedings in Life Sciences. Plant Growth Substances 1979* (*F.Skoog*, ed.), pp. *351–361*. Springer Verlag, Berlin.

Bopp, M, and Atzorn, R. (1992). The morphogenetic system of the moss protonema. *Crypt Bot* **3,** 3-10.

Bruex, A., Kainkaryam, R.M., Wickowski, Y., Kang, Y.H., Bernhardt, C., Xia, Y., Zheng, X., Wang, J.Y., Lee, M.M., Benfey, P., Woolf, P.J. and Shiefelbein, J. (2012). A gene regulatory network for root epidermis cell differentiation in Arabidopsis. *PLoS Genet* **8,** e1002446.

Chameides, W.L. and Davis, D.D. (1980). Iodineits possible role in tropospheric photochemistry. *J Geophys Res* **85,** 7383-7398

Cristina, M., Sessa, G., Dolan, L., Linstead, P., Baima, S., Ruberti, I. and Morelli, G. (1996). The *Arabidopsis* Athb-10 (GLABRA2) is an HD-Zip protein required for regulation of root hair development. *Plant J* **10,** 393-402.

Datta, S., Kim, C.M., Pernas, M., Pires, N.D., Proust, H., Tam, T., Vijayakumar, P. and Dolan, L. (2011). Root hairs: development, growth and evolution at the plant-soil interface. *Plant And Soil* **346,** 1-14.

Dolan, L., Duckett, C.M., Grierson, C., Linstead, P., Schneider, K., Lawson, E., Dean, C., Poethig, S. and Roberts, K. (1994). Clonal relationships and cell patterning in the root epidermis of *Arabidopsis*. *Development* **120,** 2465-2474.

Dharmasiri, N. and Estelle, M. (2004). Auxin signaling and regulated protein degradation. *Trends in Plant Science* **9,** 1360-1385.

Edwards, D., Duckett, J.G. and Richardson, J.B. (1995) Hepatic characters in the earliest land plants. *Nature* **374,** 635-636.

Eklund, D.M., Staldal, V., Valsecchi, I., Cierlik, I., Eriksson, C., Hiratsu, K., Ohme- Takagi, M., Sundstrom, J.F., Thelander, M., Ezcurra, I. and Sundberg, E. (2010a). The *Arabidopsis thaliana* STYLISH1 protein acts as a transcriptional activator regulating auxin biosynthesis. *Plant Cell* **22,** 349-363.

Eklund, D.M., Thelander, M., Landberg, K., Staldal, V., Nilsson, A., Johansson, M., Valsecchi, I., Pederson, E.R.A., Kowalczyk, M., Ljung, K., Ronne, H. and Sundberg, E. (2010b). Homologues of the *Arabidopsis thaliana SHI/STY/LRP1* genes control auxin biosynthesis and affect growth and development in the moss *Physcomitrella patens*. *Development* **137,** 1275-1284.

Fujita, T., Sakaguchi, H., Hiwatashi, Y., Wagstaff, S.J., Ito, M., Deguchi, H., Sato, T. and Hasebe, M. (2008). Convergent evolution of shoots in land plants: lack of auxin polar transport in moss shoots. *Evol Dev* **10,** 176-186.

Gahoonia, T.S. and Nielsen, N.E. (1998). Direct evidence on participation of root hairs in phosphorus (P-32) uptake from soil. *Plant Soil* **198,** 147-152.

Gahoonia, T.S., Care, D. and Nielsen, N.E. (1997). Root hairs and phosphorus acquisition of wheat and barley cultivars. *Plant Soil* **191,** 181-188.
Galway, M.E., Masucci, J.D., Lloyd, A.M., Walbot, V., Davis, R.W. and Schiefelbein, J.W. (1994). The *Ttg* Gene is required to specify epidermal-cell fate and cell patterning in the *Arabidopsis* root. *Dev Biol* **166,** 740-754.
Gray, W.M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001). Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins. *Nature*, 414, 271-276.
Goode, J.A., Duckett, J.G. and Stead, A.D. (1992). Protonemal morphogenesis of the moss *Tetraphis-Pellucida* Hedw in culture and in the wild. *Ann Bot*-London **70,** 519-530.
Harper, D.B., Hamilton, J.T.G., Ducrocq, V., Kennedy, J.T., Downew, A. and Kalin, R.M. (2003). The distinctive isotopic signature of plant-derived chloromethane: possible application in constraining the atmospheric chloromethane budget. *Chemosphere* **52,** 433-436.
Hiwatashi, Y., Nishiyama, T., Fujita, T. and Hasebe, M. (2001). Establishment of gene-trap and enhancer-trap systems in the moss *Physcomitrella patens*. *Plant J* **28,**105-116.
Hobe, A., Egener, T., Lucht, J., Holtorf, H., Reinhard, C., Schween, G. and Reski, R. (2004). An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene-knock-outs in a moss, *Physcomitrella patens*. *Current Gen* **44,** 339-347.
Igamberdiev, A.U. and Lea, P. (2006). Land plants equilibrate O2 and CO2 concentrations in the atmosphere. *Photosynthesis Research* **87,** 177-194.
Imaizumi, T., Kadota, A., Hasebe, M. and Wada, M. (2002). Cryptochrome light signals control development to suppress auxin sensitivity in the moss *Physcomitrella patens*. *Plant Cell* **14,** 373-386.
Jacobs, W.P. (1951). Studies on cell-differentiation - the role of auxin in algae, with particular reference to rhizoid-formation in bryopsis. *Biol Bull* **101,** 300-306.
Jang, G. and Dolan, L. (2011). Auxin promotes the transition from chloronema to caulonema in moss protonema by positively regulating *PpRSL1* and *PpRSL2* in *Physcomitrella patens*. New Phyt **192,** 319-327.
Jang G., Yi K, Pires ND, Menand B, and Dolan L. (2011)**.** *RSL* genes are sufficient for rhizoid system development in early diverging land plants. Development. **138,** 2273-2281.
Jenkins, G.I. and Cove, D.J. (1983). Phototropism and polarotropism of primary chloronemata of the moss *Physcomitrella patens* responses of mutant strains. *Planta* **159,** 432-438.
Johri, M.M. and Desai, S. (1973). Auxin regulation of caulonema formation in moss protonema. *Nature-New Biol* **245,** 223-224.
Jones, S. (2004) An overview of the basic helix-loop-helix proteins. *Genome Biol* **5,** 226.
Jones, V. A. and Dolan L. (2012). The evolution of root hairs and rhizoids. *Ann. Bot.* **110,** 205-212.
Kamisugi, Y., Cuming, A.C. and Cove, D.J. (2005). Parameters determining the efficiency of gene targeting in the moss *Physcomitrella patens*. *Nucleic Acids Res* **33**. e173
Karas, B., Amyot, L., Johansen, C., Sato, S., Tabata, S., Kawaguchi, M. and Szczyglowski, K. (2009). Conservation of *Lotus* and *Arabidopsis* basic helix-loop-helix proteins reveals new players in root hair development. *Plant Physiol* **151,** 1175-1185.
Kenrick, P. and Crane, P.R. (1997). The origin and early evolution of plants on land. *Nature* **389,** 33-39.
Kenrick, P. and Davies P. (2004). *Fossil plants*. The Natural History Museum, London.
Khalil, M.A.K. and Rasmussen, R.A. (1999). Atmospheric methyl halide. *Atmospheric Env* **33,** 1305-1321.
Kim, D., Kwon, Y.K. and Cho, K.H. (2008). The biphasic behaviour of incoherent feed-forward loops in biomolecular regulatory networks. *BioEssays* **30,** 1204-1211.
Kirik V, Simon M, Wester K, Schiefelbein J. and Hulskamp M. (2004). ENHANCER of TRY and CPC 2 (ETC2) reveals redundancy in the region-specific control of trichome development of *Arabidopsis*. *Plant Mol Biol* **55,** 389-398.
Knox, K., Grierson, C.S. and Leyser, O. (2003). AXR3 and SHY2 interact to regulate root hair development. *Development* **130,** 5769-5777.
Lee, M.M. and Schiefelbein, J. (1999). WEREWOLF, a MYB-related protein in *Arabidopsis* is a position-dependent regulator of epidermal cell patterning. *Cell* **99**, 473-483
Lee, M.M. and Schiefelbein, J. (2002). Cell pattern in the *Arabidopsis* root epidermis determined by lateral inhibition with feedback. *Plant Cell* **14,** 611-618.
Lenton, T., Crouch, M., Pires, N. and Dolan, L. (2012). First plants cooled the Ordovician. *Nat Geo*. **5,** 86-89.

Liscum E, and Reed J.W. (2002). Genetics of Aux/IAA and ARF action in plant growth and development. *Plant Mol Biol*. **49**, 387-400.

Lobert, J.M., Keene, W.C., Logan, J.A. and Yevich, R. (1999). Global chlorine emissions from biomass burning: Reactive Chlorine Emissions. *Journal of Geophysical Res* **104,** 8373-8389.

Ludwig-Muller, J., Julke, S., Bierfreund, N.M., Decker, E.L. and Reski, R. (2009). Moss (*Physcomitrella patens)* GH3 proteins act in auxin homeostasis. *New Phytol* **181,** 323-338.

Masucci, J.D. and Schiefelbein, J.W. (1994). The *rhd6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin-associated and ethylene-associated process. *Plant Physiol* **106,** 1335-1346.

Masucci, J.D., Rerie, W.G., Foreman, D.R., Zhang, M., Galway, M.E., Marks, M.D. and Schiefelbein, J.W. (1996). The homeobox gene *GLABRA 2* is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. *Development* **122,** 1253-1260.

Menand, B., Calder, G. and Dolan, L. (2007a). Both chloronemal and caulonemal cells expand by tip growth in the moss *Physcomitrella patens*. *Journal of Experimental Botany* **58,** 1843-1849.

Menand, B., Yi, K., Jouannic, S., Hoffmann, L., Ryan, E., Linstead, P., Schaefer, D.G. and Dolan, L. (2007b). An ancient mechanism controls the development of cells with a rooting function in land plants. *Science* **316,** 1477-1480.

Molkentin, J. D., Black, B., Marin, J. F. and Olson E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83**, 1125-1136.

Mravec, J., Skůpa, P., Bailly, A., Hoyerová, K., Křeček, P., Bielach, A., Petrášek, J., Zhang, J., Gaykova, V., Stierhof, Y., Dobrev, P., Schwarzerová, K., Rolčík, J., Seifertová, D., Luschnig, C., Benková, E., Zažímalová, E., Geisler, M. and Friml, J. (2009). Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature* **459**, 1136-1140.

Murre, C., McCaw. P. S., Cabrera, C., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H. and Baltmore, D. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537-544.

Nagatoshi, Y. and Nakamura, T. (2009). *Arabidopsis* HARMLESS TO OZONE LAYER protein methylates a glucosinolate breakdown product and functions in resistance to *Pseudomonas syringae* pv. *maculicola*. *The Journal of Biological Chemistry* **284,** 19301-19309.

O'Dowd, C.D., Jimenez, J.L., Bahreini, R., Flagan, R.C., Seinfeld, J.H., Hameri, K., Pirjola, L., Kulmala, M., Jennins, S.G. and Hoffmann, T. (2002) Marine aerosol formation from biogenic iodine emission. **417,** 632-636.

Penkett, S.A., Derwent, R.G., Fabian, P., Borchers, R. and Schmidt, U. (1980). Methyl chloride in the stratosphere. *Nature* **283,** 58-60.

Peter, I.S. and Davison, E.H. (2011). Evolution of gene regulatory networks controlling body plan development. *Cell* **144,** 970-985.

Peterson, R.L, and Farquhar, M.L. (1996). Root hairs: Specialized tubular cells extending root surfaces. *Bot Rev* **62,** 1-40.

Pires, N. (2010). Evolution of bHLH transcription factors that control cell differentiation in plants. PhD thesis. University of East Anglia, UK.

Pires, N. and Dolan, L. (2010). Origin and diversification of nasic-helix-loop-helix proteins in plants. *Molecular Biology and Evolution* **27,** 862-874.

Pitts, R.J., Cernac, A. and Estelle, M. (1998). Auxin and ethylene promote root hair elongation in *Arabidopsis*. *Plant J* **16,** 553-560.

Poli, D., Jacobs, M. and Cooke, T.J. (2003). Auxin regulation of axial growth in bryophyte sporophytes: its potential significance for the evolution of early land plants. *Am J Bot* **90,** 1405-1415.

Prigge, M.J., Lavy, M., Ashton, N.W. and Estelle, M. (2010). *Physcomitrella patens* auxin-resistant mutants affect conserved elements of an auxin-signaling pathway. *Current Biology* **20,** 1907-1912.

Ramos, J., Zenser, N., Leyser, O. and Callis, J. (2001). Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *The Plant Cell* **13**, 2349-2360.

Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.F., Lindquist, E.A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin-I, T., Kuroki, Y., Toyoda, A., Suzuki, Y., Hashimoto, S., Yamaguchi, K., Sugano, S., Kohara, Y., Fujiyama, A., Anterola, A., Aoki, S., Ashton, N., Barbazuk, W.B., Barker, E., Bennetzen, J.L., Blankenship, R., Cho, S.H., Dutcher, S.K., Estelle, M., Fawcett, J.A., Gundlach, H., Hanada, K., Melkozernov, A., Murata, T., Nelson, D.R., Pils, B., Prigge,

M., Reiss, B., Renner, T., Rombauts, S., Rushton, P.J., Sanderfoot, A., Schween, G., Shiu, S.H., Stueber, K., Theodoulou, F.L., Tu, H., Van de Peer, Y., Verrier, P.J., Waters, E., Wood, A., Yang, L.X., Cove, D., Cuming, A.C., Hasebe, M., Lucas, S., Mishler, B.D., Reski, R., Grigoriev, I.V., Quatrano, R.S. and Boore, J.L. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* **319,** 64-69.

Duckett, J.G., Schmid, A.M. and Ligrone, R. (1998). Protonemal morphogenesis. *In* Bryology for the Twenty-first Century. British Bryological Society, pp 223-246.

Rerie, W.G., Feldmann, K.A. and Marks, M.D. (1994). The *Glabra2* gene encodes a homeo domain protein required for normal trichome development in *Arabidopsis*. *Gene Dev* **8,** 1388-1399.

Rhew, R.C., Østergaard, L., Saltzman, E.S. and Yanofsky, M.F. (2003). Genetic control of methl halides production in *Arabidopsis*. *Current Biol* **20,** 1809-1813.

Rose, S. and Bopp, M. (1983). Uptake and polar transport of indoleacetic-acid in moss rhizoids. *Physiol Plantarum* **58,** 57-61.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M. and Leyser, O. (1998). Changes in auxin response from mutations in an *AUX/IAA* gene. *Science* **279,** 1371-1373.

Saito, T. and Yokouchi, Y. (2006). Diurnal variation in methyl halide emission rates from tropical ferns. *Atmospheric Environment* **40,** 2806-2811.

Sakakibara, K., Nishiyama, T., Sumikawa, N., Kofuji, R., Murata, T. and Hasebe, M. (2003). Involvement of auxin and a homeodomain-leucine zipper I gene in rhizoid development of the moss *Physcomitrella patens*. *Development* **130,** 4835-4846.

Schaefer, D.G. and Zryd, J.P. (1997). Efficient gene targeting in the moss *Physcomitrella patens*. *Plant J* **11,** 1195-1206.

Schaefer, D.G. and Zryd, J.P. (2001). The moss *Physcomitrella patens*, now and then. *Plant Physiol*. **127**, 1430-1438.

Schellmann, S., Schnittger, A., Kirik, V., Wada, T., Okada, K., Beermann, A., Thumfahrt, J., Jurgens, G. and Hulskamp, M. (2002). *TRIPTYCHON* and *CAPRICE* mediate lateral inhibition during trichome and root hair patterning in *Arabidopsis*. *Embo J* **21,** 5036-5046.

Simon, M., Lee, M.M., Lin, Y., Gish, L. and Schiefelbein, J. (2007) Distinct and overlapping roles of single-repeat MYB genes in root epidermal patterning. *Dev Biol* **311,** 566-576.

Sohlberg, J.L., Myrenas, M., Kuusk, S., Lagercrantz, U., Kowalczyk, M., Sandberg, G. and Sundberg, E. (2006). *STY1* regulates auxin homeostasis and affects apical-basal patterning of the *Arabidopsis* gynoecium. *The Plant J* **47,** 112-123.

Sood, S. and Hackenberg, D. (1979). Interaction of auxin, anti-auxin and cytokinin in relation to the formation of buds in moss protonema. *Z Pflanzenphysiol* **91,** 385-397.

Ståldal, V., Sohlberg, J.J., Eklund, D.M., Ljung, K. and Sundberg, E. (2008). Auxin can act independently of *CRC*, *LUG*, *SEU*, *SPT* and *STY1* in style development but not apical-basal patterning of the *Arabidopsis* gynoecium. *New Phytol* **180,** 798-808.

Thelander, M., Olsson, T. and Ronne, H. (2005). Effect of the energy supply on filamentous growth and development in Physcomitrella patens. *J Exp Bot* **56,** 653-662.

Ulmasov, T, Hagen, G. and Guilfoyle, T.J. (1997) ARF1, a transcription factor that binds to auxin response elements. *Science* **276,** 1865-1868.

Wada, T., Tachibana, T., Shimura, Y. and Okada, K. (1997). Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. *Science* **277,** 1113-1116.

Wada, T., Kurata, T., Tominaga, R., Koshino-Kimura, Y., Tachibana, T., Goto, K,. Marks, M., Shimura, Y. and Okada, K. (2002). Role of a positive regulator of root hair development, *CAPRICE*, in *Arabidopsis* root epidermanl cell differentiation. *Development* **129,** 5409-5419.

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D. and Gray, J.C. (1999). The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *The Plant Cell* **11,** 1337-1349.

Weijers, D. and Jürgens, G. (2005). Auxin and embryo axis formation: the ends in sight? *Curr Opin Plant Biol* **8,** 32-37.

Yi, K., Menand, B., Bell, E. and Dolan, L. (2010). A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. *Nat Genet* **42,** 264-267.

Yokouchi, Y., Ikeda, M., Inuzuka, Y. and Yukawa, T. (2002) Strong emission of methyl chloride from tropical plants. *Nature* **416,** 163-165.

Appendices

Appendix 1 – Primers sequences

qRT-PCR primers

PpLRL1

CGTGGTCAAGCTACTGATCCT

CGCCTTATCCGTCTTATTCG

PpLRL2

AGTATTGCTGAACGGCTTCG

CAGCACCTCCTAATCGACTCA

PpGAPDH

CTTGAGAAGCCTGCCTCCTA

TGCTGTCGGTAATGAAGTCG

PpEF1α

GGATCTTGTCGGGGTTGTA

TTTCACCTTGGGAGTGAAGC

Genotyping *Physcomitrella* HOL knock-out

p1 GCTGCAATTGGTAAGCCTCT

p2 GCCGGCCAGATCTATAACTTC

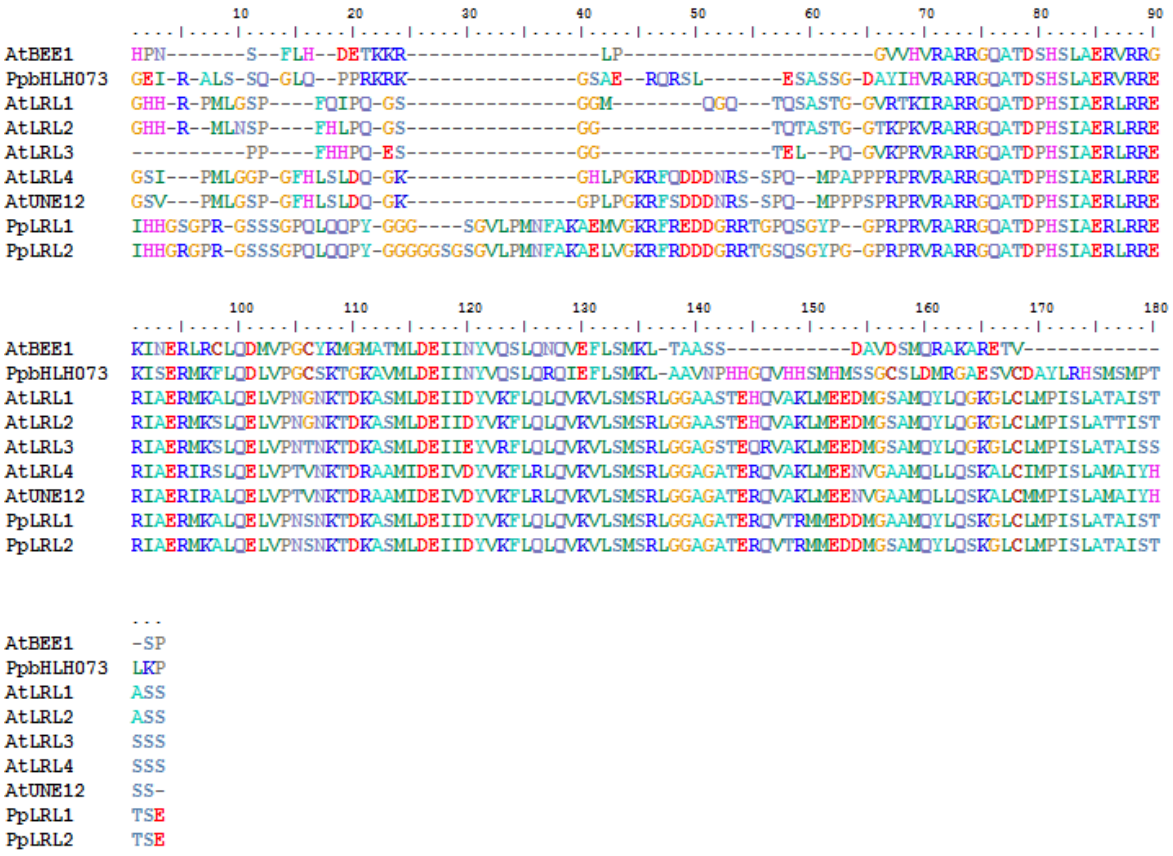
p3 GGTGGAGCTCGGTACCATAA

p4 CCATGGGAATAATAATCTTTTGGA

p5 GCATTCGGATTGTGATCCTT

p6 ACAACCCCAACAGTGGTAGC

Appendix 2 – Alignment of the C-terminus of LRL proteins

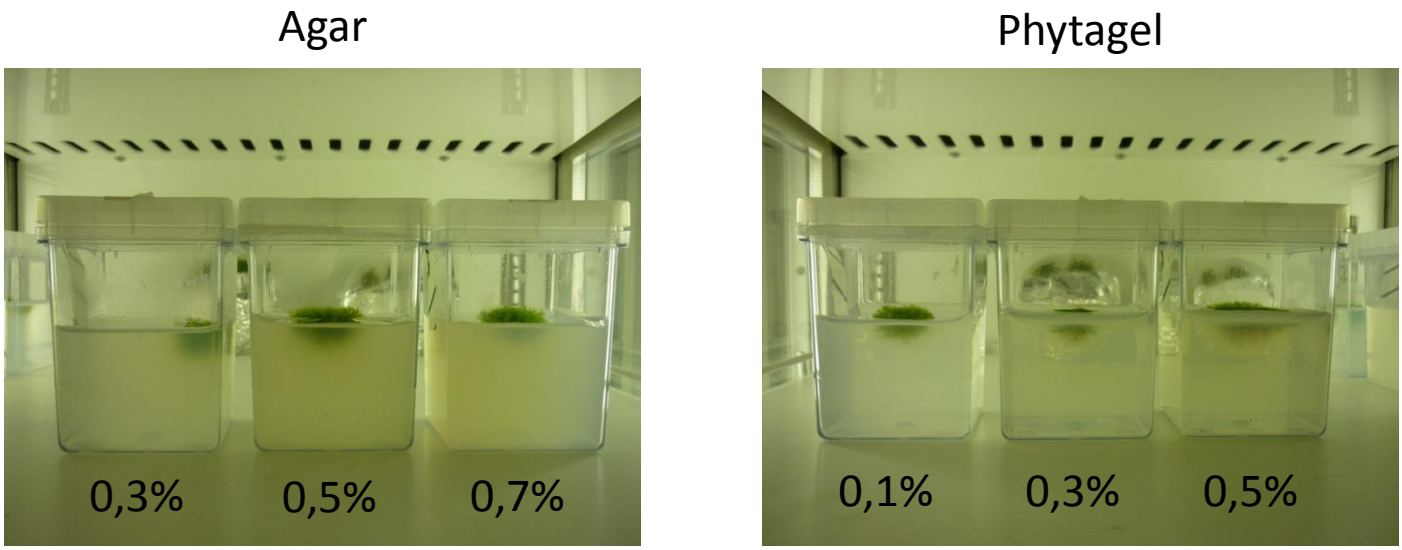


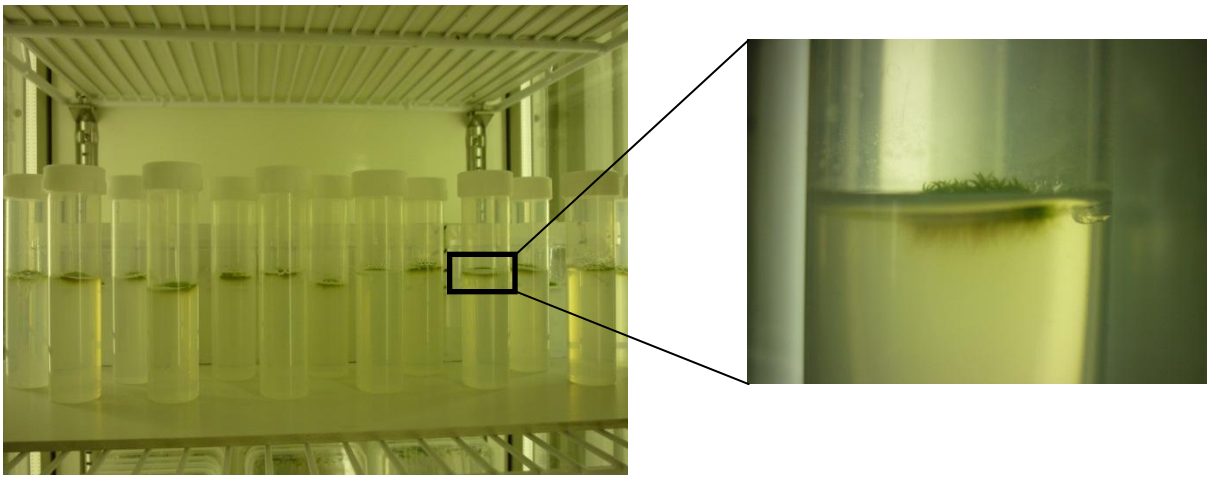
Appendix 3 – Maximum likelihood tree of *A. thaliana* and *Physcomitrella patens* LRL proteins

Appendix 3 – Maximum likelihood tree of *A. thaliana* and *Physcomitrella patens* LRL proteins.

The tree was calculated using the alignment shown in Appendix 2 and rooted with two bHLH subfamily XII proteins (AtBEE1 and PpbHLH073). Bootstrap values are indicated in the nodes.

Appendix 4 – Visualization of *P. patens* rhizoids





Appendix 4 – Visualization of *P. patens* rhizoids.

21 days old WT protonemal colony was placed into Magenta boxes with different concentrations of agar or phytagel and minimal media with 0,5% phytagel showed to be the best for rhizoids visualization (up). 21 days old WT protonemal colony was placed into tubes with minimal media with 0,5% phytagel and after five days it was possible to observe rhizoids growing from the colony.