LRL genes are ancient regulators of tip-growing rooting cell development in land plants



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Thesis submitted for the Degree of Doctor of Philosophy

Michaelmas 2013

To the fantastic people I met in Oxford.

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Abstract

Evolution of developmental genes is an important mechanism for plant morphological evolution. The *LRL* genes are an ancient group of bHLH transcription factors that positively regulate root hair development in angiosperms. Here I show that, in the moss *Physcomitrella patens*, two *LRL* genes are present and they positively regulate rhizoid and caulonema (a rhizoid-like cell type) development. GUS-transcriptional reporter plants show that both *PpLRL1* and *PpLRL2* are expressed in tissues giving rise to caulonemata. Loss-of-function mutants in either *PpLRL1* or *PpLRL2* led to defective rhizoid and caulonema development, and the *Pplrl1 Pplrl2* double loss-of-function mutants completely lack rhizoids and caulonemata. Consistent with this, gain-of-function mutants show enhanced rhizoid and caulonema development. In addition, I show that the stimulatory effects of auxin and low phosphate on the development of rhizoids and/or caulonemata required *PpLRL* gene function. Together, these results show that *LRL* genes are conserved, positive regulators in tip-growing rooting cell development in land plants. To elucidate whether *LRL* genes belong to part of a conserved gene network, I use qRT-PCR to determine the transcriptional interaction between *LRL* genes and the Class I *RSL* genes, which is another group of conserved regulators of rhizoids and root hairs. Comparing the *LRL-RSL* network between *P. patens* and *A. thaliana* reveals that *LRL* and Class I *RSL* genes are transcriptionally independent of each other in *P. patens* but one *LRL* gene is transcriptionally downstream of Class I *RSL* genes in *A. thaliana*. This suggests that the gene network controlling tip-growing rooting cell development has changed since mosses and angiosperms last shared a common ancestor.

List of abbreviations

aLRT	approximate likelihood-ratio test
ARF	Auxin Response Factor
bHLH	basic-Helix-Loop-Helix
bp	base pair
CaMV	Cauliflower mosaic virus
CDS	Coding sequence
Col-0	Columbia-0 ecotype of *Arabidopsis thaliana*
CTAB	Cetyltrimethylammonium bromide
GO	Gene Ontology
GUS	beta-glucuronidase
kb	Kilo-base pair
LRL	*Lotus japonicus ROOTHAIRLESS1-LIKE*
ML	Maximum Likelihood
Mya	Million years ago
NAA	1-Naphthaleneacetic acid
PCR	Polymerase Chain Reaction
qRT-PCR	quantitative PCR
RSL	*RHD-SIX-LIKE*
SD	Standard deviation
SE	Standard error of the mean
Ws	Wassilewskija ecotype of *Arabidopsis thaliana*
WT	Wild Type

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1 Introduction

Phylogenetic and fossil evidence suggest that land plants evolved from charophyte algae (Lewis & McCourt, 2004; Wodniok *et al.*, 2011; Finet *et al.*, 2012; Timme *et al.*, 2012). These earliest land plants probably resembled extant bryophytes and subsequent morphological innovations in different plant lineages led to the diverse morphologies observed in land plants today. These innovations include the evolution of the complex sporophyte, the vascular system, roots, leaves, seeds, and flowers.

Recent advances in molecular genetics allow us to begin to investigate the genetic basis for the evolution of major plant morphologies. This project focuses on the tip-growing, filamentous structures called root hairs found on the root epidermis in most angiosperms, and their analogous structure – the rhizoids – in rootless plants such as the bryophytes (liverworts, mosses, hornworts). Root hairs and rhizoids perform analogous functions, and are crucial for anchorage and absorption of water and nutrients. Based on the current phylogeny of the embryophytes (discussed below), rhizoids appeared first in the earlier-diverging bryophytes, and true roots (and thus root hairs) probably evolved later in the lycophytes and the angiosperms (Raven, JA & Edwards, D, 2001; Kenrick, Paul, 2002; Datta *et al.*, 2011; Jones & Dolan, 2012). From this phylogeny, one hypothesis for the evolution of the root hairs is that the genes and/or even gene regulatory networks (Davidson & Erwin, 2006; de Bruijn *et al.*, 2012) may have been recruited from the rhizoid developmental program to regulate root hair development on the root epidermis. The fact that the Class I RSL transcription factors regulate root hair development in

Arabidopsis thaliana and rhizoid development in *Physcomitrella patens* preliminarily supported this hypothesis (Menand *et al.*, 2007b; Jang *et al.*, 2011).

To determine whether it is generally true that recruitment of rhizoid development genes underpinned the evolution of the root hair, and whether there is any evidence of a conserved genetic network (i.e. conserved transcriptional regulation between homologous genes) controlling the development of both root hairs and rhizoids, I characterized another group of transcription factors encoded by the *LRL* genes (*LjRHL1-LIKE*) in the model bryophyte *P. patens*. The results of these investigations are presented in this thesis, which is divided into five chapters. This introductory chapter (**Chapter 1**) briefly summarizes our current understanding of the phylogeny of land plants and discusses the rise in the sporophyte generation during land plant evolution. I also discuss the genetic mechanism for morphological evolution, with a focus on and the evolution of root hair and rhizoids. A small section that describes the development of *P. patens* is also given at the end of this chapter. **Chapter 2** describes the expression pattern of the *P. patens LRL* genes and the phenotypes of the loss-of-function and gain-of-function mutants in *PpLRL* genes. In **Chapter 3**, I show that *PpLRL* gens mediate auxin and low-phosphate response in *P. patens*. In **Chapter 4**, I determined the interaction between the Class I *RSL* genes and the *LRL* genes in *P. patens* and also *A. thaliana*, and discuss whether there is a conserved genetic network found in the rhizoids of *P. patens* and the root hairs of *A. thaliana*. I conclude with **Chapter 5** where I give a general discussion on how this work could contribute to the field. Results from additional, preliminary experiments are given in the **Appendix**, these include RNAseq experiments to determine the downstream targets of Class I *RSL* genes and *LRL* genes in *P. patens*,

and whether the steady-state transcript level of *PpLRL* genes are affected in the strigolactone-biosynthesis mutant *Ppccd8* (Proust *et al.*, 2011).

1.1 Phylogeny of land plants and their closest living algal relative

The phylogeny of land plants provides a framework for the interpretation of morphological evolution in land plants. Recent phylogenetic analyses using molecular and/or morphological data strongly suggested that land plants (embryophytes) are monophyletic and are most closely related to the charophyte algae (Kenrick & Crane, 1997a; Lewis & McCourt, 2004; Becker & Marin, 2009; Finet *et al.*, 2010; Wodniok *et al.*, 2011; Finet *et al.*, 2012; Timme *et al.*, 2012). These studies also led to the delimitation of several monophyletic land plant lineages, including the euphyllophytes (and angiosperms and gymnosperms within it) and the lycophytes. The bryophytes, in contrast, have been shown to form a paraphyletic grade (**Figure 1-1**).

Although land plants are likely to be most closely related to the charophyte algae, there have been some uncertainties as to which charophyte lineage(s) is sister to the land plants. Morphological data suggested that either the Charales or the Coleochaetales represents the closest living relative of the land plants (Kenrick & Crane, 1997a; Karol *et al.*, 2001; Bowman, 2013). Land plants show a number of characters that they share with members of the Charales and the Coleochaetales, but not with any other orders of the charophyte algae. These include the presence of phragmoplast during cell division, the presence of plasmodesmata, and the presence of apical cells with less than three cutting edges (Bowman, 2013). However, phylogenetic analyses using nuclear gene sequences or chloroplast genomes resolved either Zygnematales or a clade containing Zygnematales and

Coleochaetales as the sister lineage to the embryophytes (Turmel *et al.*, 2006; Wodniok *et al.*, 2011; Finet *et al.*, 2012; Timme *et al.*, 2012). The closest sister lineage(s) to the land plants thus remains unresolved (**Figure 1-1**).

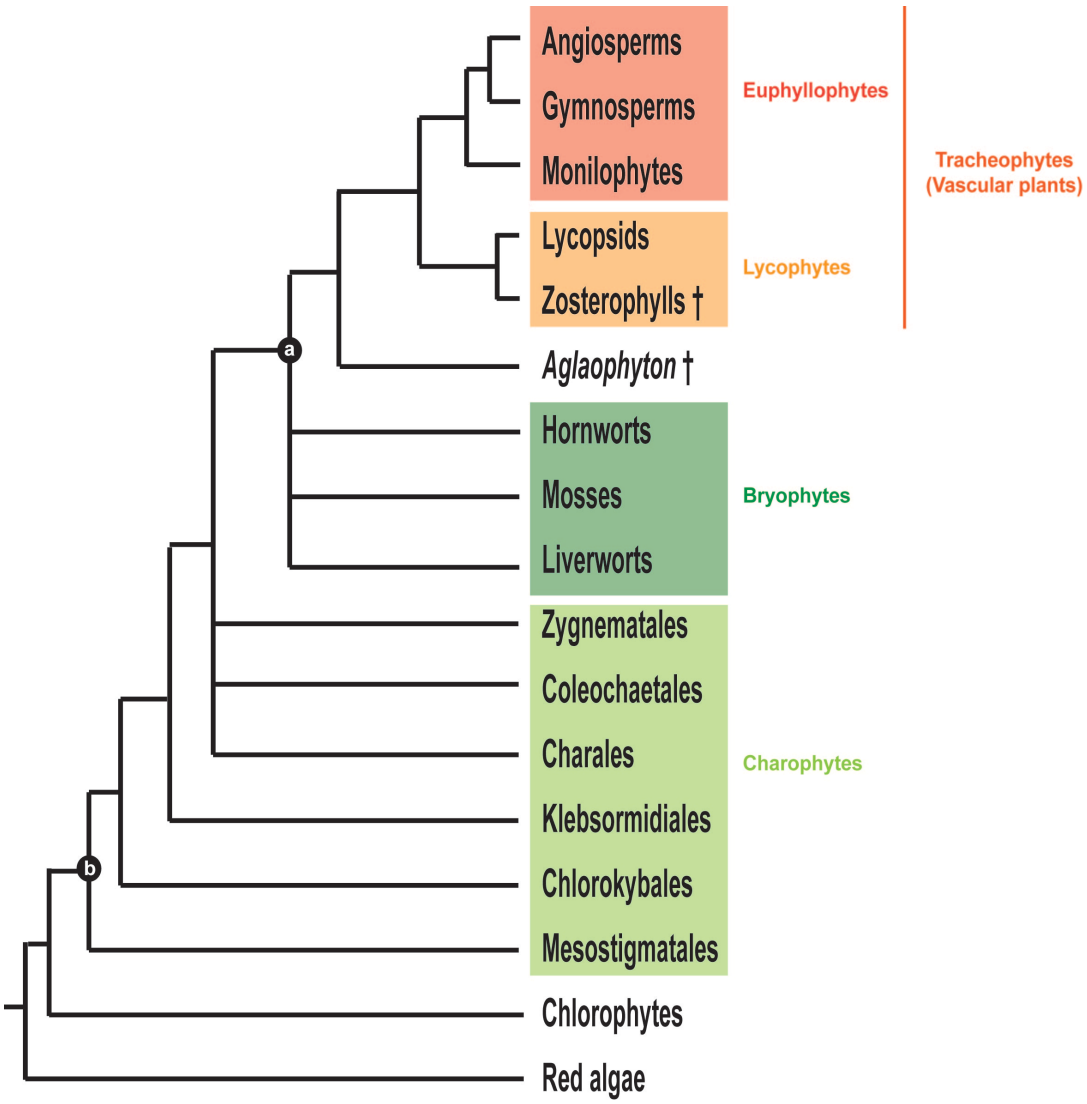


Figure 1-1 Phylogeny of land plants (Kenrick & Crane, 1997b; Lewis & McCourt, 2004; Becker & Marin, 2009; Graham *et al.*, 2009; Finet *et al.*, 2010; Wodniok *et al.*, 2011; Finet *et al.*, 2012; Timme *et al.*, 2012; Bowman, 2013). Node a represent the last common ancestor of land plants, estimated at ~450 Mya (Clarke *et al.*, 2011); node b represent the last common ancestor of the (land plants + charophyte algae, i.e. streptophytes clade)(Becker & Marin, 2009), estimated at ~630-850 Mya (Parfrey *et al.*, 2011). Note that molecular clock analyses are subject to considerable variation and should be interpreted carefully (Kenrick, 2011).

1.2 *KNOX* genes, polycomb proteins, and the evolution of the multicellular sporophyte

One of the most important synapomorphies of embryophytes is the presence of a multicellular, diploid sporophyte embryo (Kenrick & Crane, 1997a). In charophyte algae (including Zygnematales, Coleochaetales, and Charales), the diploid sporophyte stage is limited to the single fertilized egg cell (the zygote), which undergo meiosis to produce four haploid spores (Niklas & Kutschera, 2009). These spores germinate to produce the multicellular, haploid gametophyte, which produces male and female gametes by mitosis; the gametes fuse to produce the zygote, completing the life cycle (Niklas & Kutschera, 2009). In other words, in charophyte algae, only the haploid gametophyte is multicellular, and such a life cycle has been described as haplobiontic-haploid (Niklas & Kutschera, 2009). In contrast, both the haploid gametophyte and the diploid sporophyte is multicellular in embryophytes; they thus exhibit a diplobiontic life cycle i.e. a life cycle where there is an alternation of multicellular gametophyte and sporophyte generations.

The origin of the multicellular sporophyte in the embryophytes was one of the most important evolutionary transitions in the embryophytes, and has been discussed extensively by botanists since the late nineteenth century (Haig, 2008). Traditionally, two theories have been put forward to explain the origin of the multicellular sporophyte (Blackwell, 2003; Haig, 2008; Niklas & Kutschera, 2009; Gerrienne & Gonez, 2010; Qiu *et al.*, 2012). The homologous theory postulated that land plants were derived from a green alga with an isomorphic alternation of generations, such as *Ulva*. The dominance of the gametophyte observed in bryophytes was postulated to be the result of a reduction of the sporophyte, whereas the dominance of the sporophyte in vascular plants was due to the reduction of the

gametophyte generation. On the other hand, the antithetic (or interpolation) theory, suggests that land plants were derived from a green alga with a haplobiontic-haploid life cycle, as observed in extant charophyte algae. According to this theory, the origin of the multicellular sporophyte was due to delayed meiosis and subsequent mitotic cell division of the zygote, and the retention of the zygote within the archegonia. Our current understanding of the phylogeny of land plants (**Figure 1-1**) strongly supports the antithetic theory because the charophyte algae, which lacks a multicellular sporophyte generation, has been shown to be more closely related to land plants than chlorophyte algae such as *Ulva*. This suggests that a diploid multicellular sporophyte probably evolved due to the elaboration of the zygote in the ancestor of land plants. In the most basal land plants such as the bryophytes, the sporophyte, even though it is multicellular, remained small and is dependent on the larger, and morphologically more complex, gametophyte. In contrast, the vascular plants possess a sporophyte generation that is larger in size and greater in morphological complexity, and the gametophyte are greatly reduced, as exemplified by pollens and the embryo sac in angiosperms. Thus, the multicellular land plant sporophyte likely evolved from an ancestral, unicellular zygote.

The genetic mechanisms that regulate the development of a multicellular sporophyte may have evolved from genes that regulate the sexual cycle in the ancestor of land plants (Sakakibara *et al.*, 2013). In the unicellular chlorophyte algae *Chlamydomonas*, which is sister to land plants plus charophyte algae (**Figure 1-1**), zygote development is regulated by two homeoproteins: Gsm1 (Gamete-specific *minus*1, encoded by the minus mating type gamete) and Gsp1 (Gamete-specific *plus*1, encoded by the plus mating type gamete)(Lee *et al.*, 2008). Gsm1 belongs to the KNOX class of homeoproteins, and Gsp1 is distantly related to the

BELL class of homeoproteins (Lee *et al.*, 2008). Upon gamete fusion, heterodimerization of Gsm1 and Gsp1 led to the development of the zygote (Lee *et al.*, 2008). Plus-type mutants that lack the a functional Gsp1 resulted in defective zygote development after mating with minus-type gametes (Nishimura *et al.*, 2012); whereas ectopic expression of the Gsp1 protein in the minus mating type gamete led to zygote development without gamete fusion (Zhao *et al.*, 2001). These results demonstrated the importance of this ancestral homeoprotein interaction (which shows sequence homology with the KNOX/BELL proteins) in regulating the haploid-to-diploid transition in the chlorophyte algae. In land plants, KNOX genes are divided into two sub-families: KNOX I and KNOX II (Mukherjee *et al.*, 2009). In the moss *Physcomitrella patens*, the KNOX II genes *PpMKN1* and *PpMKN6* regulate the haploid gametophyte to diploid sporophyte transition by repressing haploid-specific development in the sporophyte (Sakakibara *et al.*, 2013). *Ppmkn1 Ppmkn6* double loss-of-function mutants resulted in the diploid sporophyte embryo developing a morphology that resembles the haploid gametophyte generation (Sakakibara *et al.*, 2013). On the other hand, KNOX I genes regulate sporophyte meristem development in land plants, but are not involved in haploid gametophyte development (Sano *et al.*, 2005; Singer & Ashton, 2007; Sakakibara *et al.*, 2008). Together, these results suggest that the KNOX genes, and potentially KNOX-BELL interactions (Sakakibara *et al.*, 2013), have played crucial roles in the evolution of the multicellular sporophyte. In addition, genes of the Polycomb Repressive complex 2 may also be important in regulating the haploid-to-diploid transition in land plants (Mosquna *et al.*, 2009; Okano *et al.*, 2009).

1.3 Genetic mechanisms underpinning the evolution of morphological complexity in land plants

One mechanism to explain the elaboration of the sporophyte during land plant evolution is that genes that regulate the development of the morphological characters in the gametophyte generation have been recruited to the sporophyte generation, leading to the development of complex characters in the sporophyte. The fact that the Class I *RSL* genes regulate rhizoid and caulonema development in the gametophyte of *P. patens* and root hair development in *A. thaliana* supports this hypothesis (Menand *et al.*, 2007b). An additional mechanism that could contribute to the elaboration of the sporophyte is the neo-functionalization of sporophytic genes. For example, Class I KNOX genes control sporophyte development in both *P. patens* and angiosperms, but do not control gametophyte development in *P. patens* (Sano *et al.*, 2005; Singer & Ashton, 2007; Sakakibara *et al.*, 2008). General mechanisms that generate genetic diversity, such as gene (and genome) duplication, and changes in gene regulatory networks due to evolution of *cis*-regulatory elements, has no doubt also play a role in the evolution of land plant morphologies (Erwin & Davidson, 2009; Rosin & Kramer, 2009; Booth *et al.*, 2010; Soyer, 2010; de Bruijn *et al.*, 2012; Monteiro, 2012).

1.4 Evolution of root hairs and rhizoids

Rhizoids and root hairs are functionally analogous structures, and are usually found in different stages of the plant life cycle. Rhizoids are found in the gametophyte of bryophytes and lycophytes, whereas root hairs are found on the root epidermis in the sporophyte generations of vascular plants. Tip-growing rooting cells that are found in the sporophyte generation but not on the root are also sometimes referred to as rhizoids. While the functions of rhizoids are less-well characterized, root hairs

are known to be crucial for water and nutrient uptake, anchorage, and interaction with soil microorganisms.

The phylogeny of land plants suggest that rhizoids first evolved in the gametophyte of land plants, then, as the sporophyte rose to dominance, tip-growing rooting cell appeared in the sporophyte generation, and are referred to as root hairs as true roots evolved (Raven, JA & Edwards, D, 2001; Kenrick, Paul, 2002; Datta *et al.*, 2011; Jones & Dolan, 2012).. This is summarized in **Figure 1-2** and is described briefly in the section below.

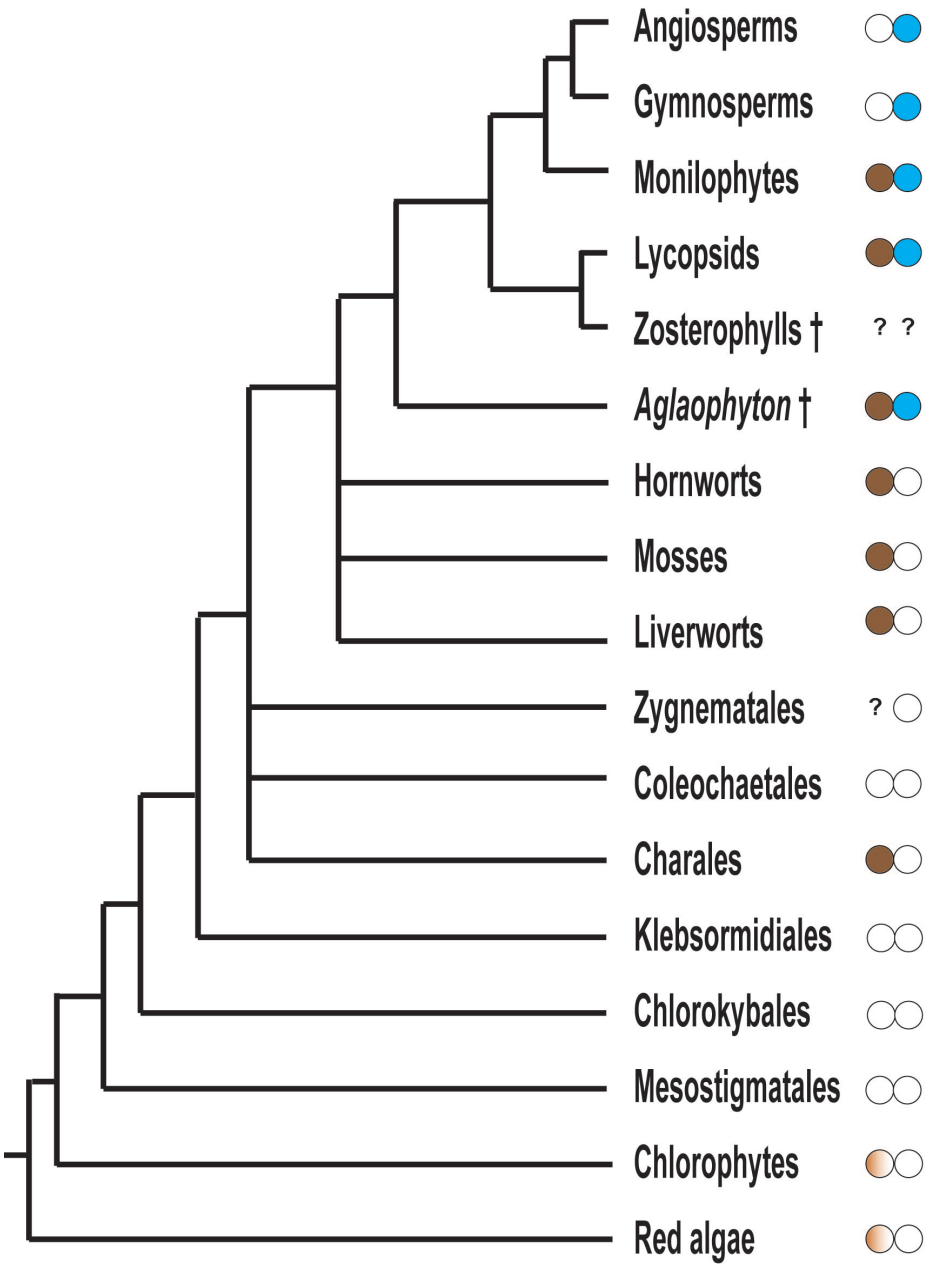


Figure 1-2 Evolution of rhizoids and root hairs. Brown dots indicate the presence of rhizoid in the gametophyte generation; blue dots represent presence of root hairs/rhizoids in the sporophyte generation. Empty dots represent the absence of rhizoids/ root hairs. Half filled dots indicate the presence of rhizoids in only a small minority of species in that lineage, which are probably results of convergent evolution (see text for details). Question marks (?) indicate that data is not available.

1.4.1 Rhizoids in the closest living relatives of land plants

Among the three potential sister groups to land plants – Charales, Coleochaetales and Zygnematales – only Charales and some species of the Zygnematales possess rhizoids.

Chara spp. develops rhizoids that are filamentous and morphologically similar to rhizoids in land plants, (Braun, 1997; Feist *et al.*, 2005; Graham *et al.*, 2009). Although nutrient absorption occurs throughout the plant body, including the green shoots (Littlefield & Forsberg, 1965; Vermeer *et al.*, 2003), *Chara* rhizoids are likely to contribute significantly to absorption from the sediment, which has three orders of magnitude higher concentration of soluble phosphate than the bulk water phase surrounding the rest of the plant (Box, 1986). *Chara* rhizoids have also been shown to be more efficient in the uptake of nitrogen (in nitrates and ammonium) than the plant taken as a whole (Box, 1987). The rhizoids in *Chara*, which show positive gravitropism, also function to anchor the thallus to the sediment (Braun & Limbach, 2006). Together these data indicate that *Chara* rhizoids are functionally analogous to filamentous rooting cells like root hairs and rhizoids in land plants.

Rhizoids are also found in the some filamentous species of Zygnematales. The Zygnematales forms a monophyletic group and members of the Zygnematales are either unicellular or are composed of unbranched filaments (Graham *et al.*, 2009). In the filamentous genus *Spirogyra*, rhizoids sometimes develop from the terminal cell of a filament and serve to anchor the algae to the substrate (Inoue *et al.*, 2002). A rhizoid primordium forms from the terminal cell of a filament, which then differentiates into a filamentous ("rod-like") rhizoid that is either branched or unbranched (Nagata, 1973). At this stage the rod-like rhizoids superficially

resemble unicellular rhizoids or root hairs in land plants. However, depending on the growth condition (Nagata, 1979; Ikegaya *et al.*, 2008), the rhizoid primordium or the rod-like rhizoid can also differentiate into a rosette-shaped "rhizoid", without unidirectional elongation of the primordial protrusion, and develop into a structure that resembles "a flower of chrysanthemum" (Nagata, 1973). In the filamentous genus *Zygnema*, some species also produce either unicellular or multicellular rhizoidal outgrowths that probably function in attachment (Stancheva *et al.*, 2012). It is unclear whether these rhizoids share the same genetic components as those in land plant rhizoids / root hairs and thus it is unclear whether these rhizoids share the same evolutionary origin as those in land plants. However, rhizoids found in other algae, such as the red algae *Polysiphonia* (Meñez, 1964) and the chlorophyte green algae *Valonia* (Elvira et al., 2012) and *Fritschiella* (Randhawa, 1946), are likely the results of convergent evolution because most other lineages of red algae or chlorophyte green algae do not possess rhizoids.

1.4.2 Rooting structures in the bryophytes

The bryophytes (liverworts, mosses, hornworts) represent a grade that consists of living representatives of the earliest-diverging land plants, but the relatively sparse fossil record of the bryophytes means that there is few undisputed fossil evidence for the presence of rhizoids in the earliest land plants. However, rhizoids are found in nearly all-extant bryophytes. In thalloid liverworts, unicellular rhizoids develop at the ventral surface of the thallus. Liverwort rhizoids may be colourless or pigmented and are important for anchorage and establishing mycorrhizal associations (Russell & Bulman, 2005; Goffinet & Shaw, 2009). In many complex thalloid liverworts such as *Marchantia polymorpha*, two types of rhizoids are present: smooth and pegged (O'Hanlon, 1926; Vanderpoorten & Goffinet, 2009),

and both types appear to be important for water transport (Jones & Dolan, 2012). In mosses, rhizoids are distinctively multicellular, in contrast to liverworts or hornworts, and develop from the gametophore – a leaf-bearing, shoot-like axis that produces the antheridia and the archegonia. In addition to rhizoids, many mosses also possess a specialized, tip-growing filamentous cell type called the caulonema during the early stage of their development; and there are evidence that rhizoids and caulonema are cytologically and morphologically similar structures that are controlled by similar genetic components (See below). Hornworts possess smooth unicellular rhizoids that develop on the ventral surface (Renzaglia *et al.*, 2008).

1.4.3 Rhizoids are found in the gametophyte and sporophyte generation in protracheophytes fossils in the Rhynie chert

The earliest unequivocal evidence of rhizoids came from early Devonian (ca. 400 Ma) protrancheophyte fossils such as *Horneophyton* and *Aglaophyton* from the Rhynie Chert in Scotland (Kenrick, Paul, 2002) . Unicellular rhizoids are found on prostrate stem (rhizome) in these plants and appear to develop from epidermal cells on a raised area produced by underlying hypodermal cells. The presence of arbuscular mycorrhizae in the rhizome of *Aglaophyton major* also indicates an early origin of fungal symbiosis (Brundrett, 2002). Rhizoids are found in both the sporophyte and the gametophyte stage, e.g. in *Aglaophyton major* (sporophyte) / *Lyonophyton rhyniensis* (gametophyte), and *Rhynia gwynne-vaughanii* (sporophyte) / *Remyophyton delicatum* (gametophyte) (Kerp *et al.*, 2004; Taylor *et al.*, 2005; Taylor *et al.*, 2009). Since the sporophyte gradually increases in size and morphological diversity during the evolution of land plants, these fossils may provide a snapshot of the evolutionary event when the recruitment of the rhizoid

development mechanism from the gametophyte to the sporophyte may have contributed to the evolution of rhizoids, and later root hairs in the sporophyte.

1.4.4 Rooting structures in the lycophytes

The bryophytes and protracheophytes described above possess rhizoids but none of these plants show a shoot/root distinction in their plant body. In the protracheophytes such as *Agalophyton*, the rhizomes can be regarded as a prostrate stem that functions physiologically as a root, as no developmental differences can be found between the rhizome and the rest of the stem system (Raven, JA & Edwards, D, 2001). True roots evolved independently at least twice, once in the lycophyte lineage and once in the euphyllophytes (Raven, J & Edwards, D, 2001; Kenrick, P, 2002; Jones & Dolan, 2012). An early indication of this distinction in the lycophyte lineage may come from the lycopsid fossil *Asteroxylon mackiei* that show a distinct contrast between its aerial stem, which had leaf-like appendages, the rhizome was leafless and had a vascular system different from that of the ariel stem (Kenrick, P, 2002). However, there is no evidence for the presence of rhizoids or root hairs (Kenrick, P, 2002). In fact, undisputed rhizoids or root hairs are generally not found in Zosterophylls or lycopsid fossils e.g. S*tigmaria* (Stewart, 1947; Gensel & Edwards, 2001). This may be due the poor preservation of rhizoids during fossilization of these plants. The Zosterophyll *Trichopherophyton teuchsanii*, for example, possess filamentous structures on the rhizome (or root) that might be interpreted as rhizoids (or root hairs) (Lyon & Edwards, 1991).

Root hairs are found in all three extant families of lycophytes (Lycopodiaceae, Isoeteaceae, and Selaginellaceae). In *Selaginella,* a unique, leafless and hairless axis called the rhizophore gives rise to the root, which possesses a root cap and

numerous root hairs (Lu & Jernstedt, 1996; Banks, 2009; Schulz *et al.*, 2010; Otręba & Gola, 2011). In *Isoetes*, the lower part of the corm (the rhizomorph) gives rise to the root, which possesses numerous root hairs (Yi & Kato, 2001; Ctvrtlikova *et al.*, 2009). Numerous filamentous rhizoids also develop from gametophyte tissues surrounding the spores after fertilization in *Isoetes* (Ctvrtlikova *et al.*, 2009; Ctvrtlikova *et al.*, 2012). The fact that environmental factors such as pH and aluminium concentration affect root hair (sporophytic) and rhizoid (gametophytic) development similarly (Ctvrtlikova *et al.*, 2009) suggests that their development may be controlled by similar genetic mechanisms. Root hairs are also found in members of the Lycopodiaceae (Stokey, 1907).

1.4.5 Rooting structures in the euphyllophytes

Root hairs are found in almost all euphyllophytes. In ferns, tip-growing rooting cells (rhizoids and root hairs) are found in both the gametophyte and sporophyte generation (Pearson, 1969; Banks, 1999; Racusen, 2002). Root hairs are also found in horsetails and gymnosperms (Dittmer & Reinhart, 1948; Dittmer, 1949). In angiosperms, the presence of root hairs and the pattern of root hair development on the root epidermis are well documented and three types of root epidermal pattern were recognized (Leavitt, 1904; Clowes, 2000; Dolan & Costa, 2001; Pemberton, 2001; Karas *et al.*, 2005; Datta *et al.*, 2011; Kim & Dolan, 2011). Root hairs may be produced on morphologically identical cells on the epidermis (Type 1, e.g. *Oryza sativa* and *Lotus japonicus*), alternate along a cell file along the axis of the root due to asymmetric, transverse cell division in the epidermis (Type 2, e.g. *Brachypodium distachyon*), or develop in single files along the root axis, such that hair files and non-hair files alternate along the circumference of the root epidermis (Type 3, e.g. *Arabidopsis thaliana*). In *A. thaliana*, root hairs develop from the base of the

hypocotyl and also from the root (Dolan *et al.*, 1994). In the root, hair-forming cells (trichoblasts) are found lying on top of the anticlinal cell walls of underlying cortex cells, whereas non-hair-forming cells (atrichoblasts) are found directly on top of cortex cells (Dolan *et al.*, 1994). Because the root axis have on average 8 cortical cells along the circumference of the root axis, there are on average 8 files of hair cells alternating with 1 or 2 non-hair cell files between them, producing the type 3 pattern (Dolan *et al.*, 1994).

1.5 Genetic basis for the evolution of rhizoids and root hairs in land plants

The genes and gene regulatory network regulating the different stages of root hair development in *A. thaliana* has been extensively reviewed elsewhere (Bibikova & Gilroy, 2003; Ishida *et al.*, 2008; Datta *et al.*, 2011; Bruex *et al.*, 2012; Kwasniewski *et al.*, 2013). Among the many factors involved in root hair development, transcription factors belonging to the basic helix-loop-helix (bHLH) family appear to play a particularly important role (**Figure 1-3**). This includes the Class I and Class II RSLs (Menand *et al.*, 2007b; Yi *et al.*, 2010; Jang *et al.*, 2011; Pires *et al.*, 2013), TMO5/bHLH32 (Chen *et al.*, 2007; Schlereth *et al.*, 2010) and the LRLs (see below). Several bHLHs also play a role in root development and epidermal patterning, including GL3, EGL3, and TMO7 (Bernhardt *et al.*, 2003; Zhang *et al.*, 2003; Bernhardt *et al.*, 2005; Schlereth *et al.*, 2010). Here I focus on the LRLs because they control root hair development not only in *A. thaliana* but also in *Lotus japonicus* and *Oryza sativa* (Ding et al., 2009; Karas et al., 2009), despite the different root epidermal pattern found in these plants.

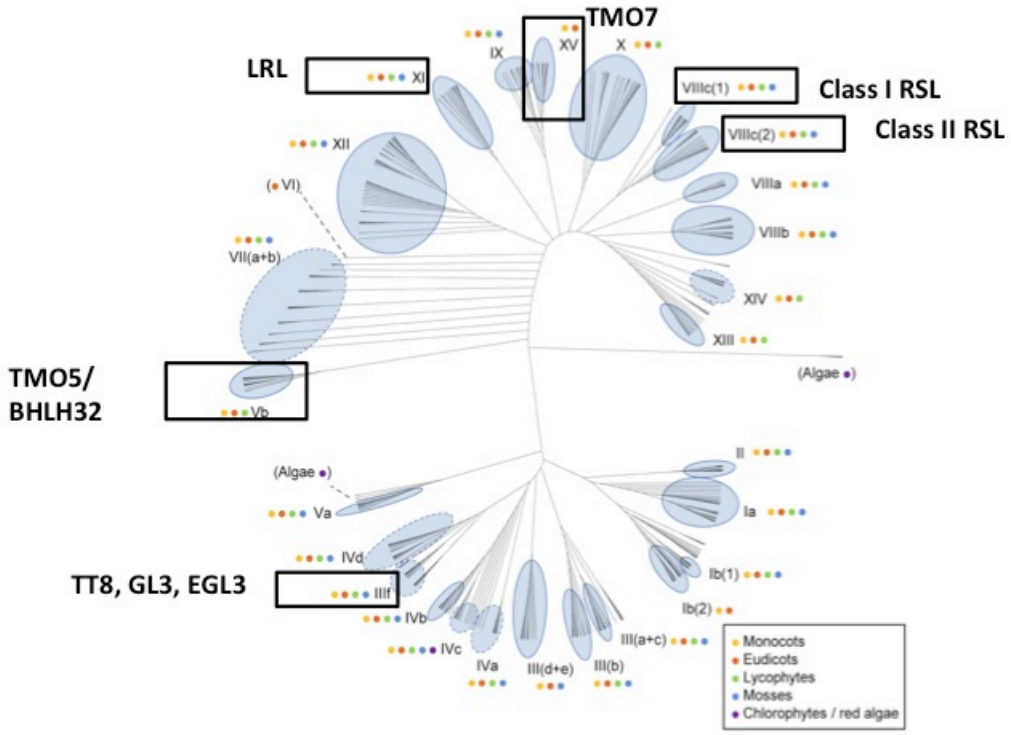


Figure 1-3 A Maximum likelihood analysis if plant bHLH proteins shown as an unrooted cladogram, modified from (Pires & Dolan, 2010). Members that are involved in rooting system development are indicated. Blue shadings delineate the 26 subfamilies of plant bHLHs. Coloured dots show the species in which the subfamilies can be found. This thesis focuses on the LRL proteins, and their interactions with the Class I RSLs. For details see text.

The LRLs comprise subfamily XI of plant bHLH transcription factors and are characterized by the presence of a conserved motif in addition to the bHLH domain (Pires & Dolan, 2010). In *L. japonicus,* loss-of-function mutation in the *Lotus japonicus ROOTHAIRLESS1 (LjRHL1)* gene resulted in the almost complete loss of root hairs and the root epidermis appears smooth with no bulges (Karas *et al.*, 2005; Karas *et al.*, 2009). In *A. thaliana,* plants lacking in *AtLRL1, AtLRL2 and/or AtLRL3* gene function develops fewer, shorter, or abnormal hairs; and the Atlrl1+/- *Atlrl2-/- Atlrl3-/-* plants develop almost no root hairs (**Figure 1-4**) (Karas *et al.*, 2009; Bruex

et al., 2012). In *Oryza sativa*, loss-of-function mutation in the *OsRHL1* gene resulted in shorter root hair compared to WT while overexpression of *OsRHL1* resulted in longer root hairs than WT. Together, these data suggest that these *LRL* genes are conserved components of root hair development in diverse angiosperms showing different root epidermal patterns, and that the role of *LRL* genes in controlling root hair development may have evolved independently of the mechanisms controlling root epidermal pattern.

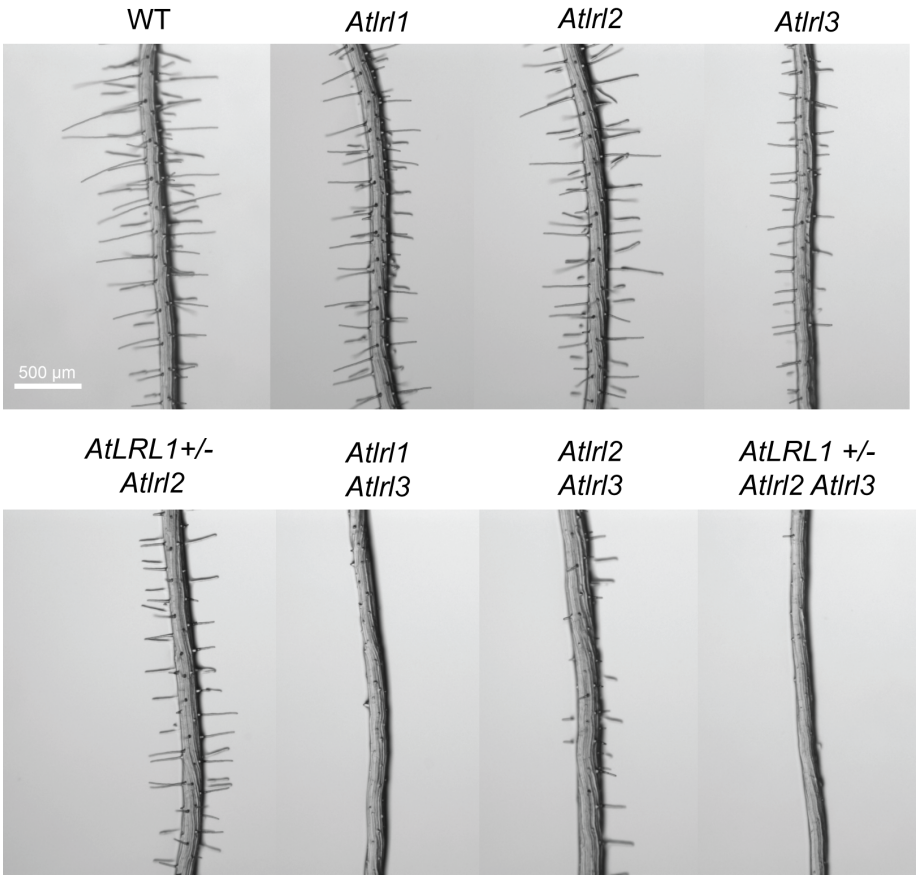


Figure 1-4 Loss-of-function mutations in *A. thaliana LRL* genes causes defective root hair development. My observations are consistent with the published literature (Karas *et al.*, 2009; Bruex *et al.*, 2012). *Atlrl1, Atlrl2*, and *Atlrl3* single mutant plants all show shorter root hairs compared to wild type, but the *Atlrl3* mutants have hairs that are shortest among the three single mutants, while *Atlrl2* mutants have the least severe root hair defect phenotype. Together with the fact that *Atlrl1 Atlrl3* has a more severe root hair defect phenotype than *Atlrl2 Atlrl3*, these results show that *AtLRL1* is more important than *AtLRL2* in regulating root hair development, while *AtLRL3* appears to be the most important regulator among these three genes. The *AtLRL1+/- Atlrl2* mutant – with T-DNA inserts heterozygous for *AtLRL1* and homozygous for *AtLRL2* – show a root hair defect that is more severe than *Atlrl1* or *Atlrl2* mutants (this line was used because the *Atlrl1 Atlrl2* double mutant cannot be recovered) (Karas *et al.*, 2009). This suggests that *AtLRL1* and *AtLRL2* have overlapping function in controlling root hair development. The *AtLRL1+/- Atlrl2 Atlrl3* "triple" mutant develops very few hairs and the root epidermis appears smooth with few bulges. The seeds were grown vertically on Murashige & Skoog medium, 1% sucrose, and 0.5% Phytagel (Sigma, UK) at pH 5.8 in a SANYO MLR-351 growth chamber at 25˚C and a 16 h light / 8 h dark regime, after being stratified on media at 4˚C in the dark for 3-5 days. T-DNA lines used are as described previously(Karas *et al.*, 2009). The double and triple mutant seeds were kindly provided by Dr. Krzysztof Szczyglowski (Agriculture and Agri-Food Canada).

In *A. thaliana*, the Class I *RSL* genes (Menand *et al.*, 2007b) and the Class II *RSL* genes (Yi *et al.*, 2010; Pires *et al.*, 2013) also control root hair development; and there is evidence suggesting that at least one *LRL* genes may interact with the Class I *RSL* genes in *A. thaliana* (Karas *et al.*, 2009; Bruex *et al.*, 2012) (**Figure 1-5**) *AtRHD6* are expressed in hair cells and the *Atrhd6 Atrsl1* double loss-of-function mutants are nearly hairless (Menand *et al.*, 2007b). The function *AtRHD6* and *AtRSL1* are mediated, at least in part, through another group of closely related genes called the Class II *RSL* genes, which includes *AtRSL2, AtRSL3, AtRSL4,* and *AtRSL5* (Pires et al., 2013). *AtRSL4* are transcriptionally downstream of the Class I *RSL* genes and the *Atrsl4* loss-of-function mutants have less and shorter root hairs than wild type (Yi *et al.*, 2010). Loss-of-function mutation in *AtRSL2* enhances this defect in the *Atrsl4* background and the *Atrsl2 Atrsl4* double mutants are hairless (Yi *et al.*, 2010). Root hairs in plants harboring a *35S:RSL4* construct are much longer than wild type plants (Yi *et al.*, 2010). Low phosphate treatment, which induces root hair development, also led to increased *RSL4* expression, suggesting that low phosphate induces root hair development by up-regulating *AtRSL4* (Yi et al., 2010). Auxin treatment, which induces root hair development in wild type plants, can partially rescue the defective root hair phenotype of *Atrhd6 Atrsl1* mutants but not that of the *Atrsl2 Atrsl4* mutants; auxin treatment also positively regulates the expression of *AtRSL4* (Yi et al., 2010). RT-PCR and microarray data suggest that steady-state levels of *AtLRL3* (but not *AtLRL1* or *AtLRL2*) mRNA decreased in the *Atrhd6 Atrsl1* mutants (Karas *et al.*, 2009; Bruex *et al.*, 2012), indicating that Class I *RSL* genes positively regulate *AtLRL3* expression, but do not regulate the expression of *AtLRL1* or *AtLRL2*. Microarray data also suggests that auxin positively regulates the expression of *AtLRL3* (Bruex *et al.*, 2012). However,

the interaction between *AtLRL* genes and Class II *RSL* genes such as *AtRSL4* is unclear. It is therefore possible that auxin induces *AtLRL3* expression via *AtRSL4*. Together these results led to a model of root hair growth by a gene network consisting of auxin, low phosphate, Class I and Class II *RSL* genes, and the *LRL* genes (**Figure 1-5**). A more elaborate model focusing on auxin and the *RSL* genes (Class I and II) has been published previously (Pires *et al.*, 2013).

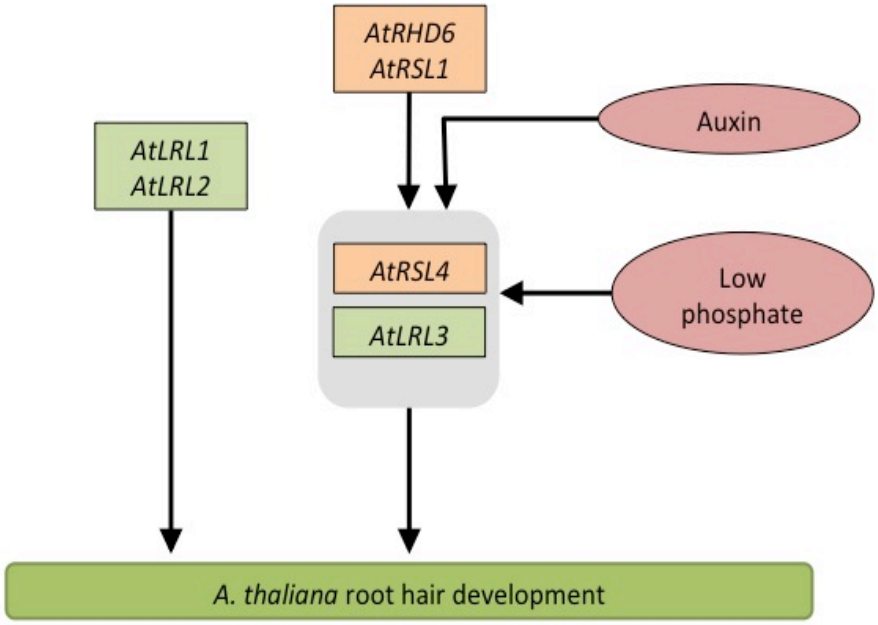


Figure 1-5 A model of the gene network regulating root hair development in *A. thaliana*. Arrows represent positive transcriptional regulation. This model focuses on the Class I and Class II *RSL* genes and the *LRL* genes and is not exhaustive. Note that *AtRSL4* and *AtLRL3* are clustered together here because the transcriptional regulations between them are not clear, even though they are both regulated by *AtRHD6/AtRSL1* and auxin. Low phosphate positively regulates *AtRSL4* but its effect on *AtLRL3* is unclear. *AtLRL1* and *AtLRL2* appears to act independently of *AtRHD6* and *AtRSL1* to control root hair development (Karas *et al.*, 2009; Bruex *et al.*, 2012), but their interactions with auxin and low phosphate have not been clearly demonstrated.

The fact that *RSL* genes also control tip-growing rooting cell development in the moss *P. patens* (Menand *et al.*, 2007b; Jang & Dolan, 2011; Jang *et al.*, 2011; Pires *et al.*, 2013) led to the hypothesis that *LRL* genes and *RSL* genes may together represent an ancient gene regulatory network that controls the development of tip-growing rooting cells in land plants. In the following chapters, I will test this hypothesis by characterizing the *PpLRL* genes and their interaction with other factors regulating tip-growing rooting cell development in *P. patens*, including auxin, low phosphate, and the Class I *RSL* genes.

1.6 Life cycle of *P. patens*

Physcomitrella patens (Hedw.) Bruch & Schimpis, also known as *Aphanorrhegma patens* (Hedw.) Lindb., is a moss in the family Funariaceae (Cox, 1999). It is widely distributed across Europe, Scandinavia, and North America, but is not a common species (Smith, 2004; Cove, 2005). They are usually found growing by wet areas such streams, wet tracks and ditches (Smith, 2004). *P. patens* is one of the most widely used model system for studies in mosses (Reski, 1998; Cove, 2005; Cove *et al.*, 2006) and is the first among the bryophytes to have its genome published (Rensing *et al.*, 2008). Its haploid-dominant life cycle and the relative ease of gene disruption by homologous recombination (Schaefer, 2001) make it a good model system for genetic analysis.

The life cycle of *P. patens* (**Figure 1-6**) begins with the germination of the haploid spore. The first cell type to grow from a germinating spore is chloronema in identity. As a colony grows and the protonema filaments extend further away from the centre of the colony, apical cells of chloronema filaments begin to differentiate into caulonema (Duckett *et al.*, 1998). Together, the chloronema and the caulonema

constitute the protonema. A fully differentiated caulonema filament can be distinguished from a chloronema filaments by its oblique cell walls, low chloroplast density and higher growth rate of 20 μm per hour (chloronema has perpendicular cell walls, is chloroplast-rich and grows at 6 μm per hour) (Duckett *et al.*, 1998; Menand *et al.*, 2007a; Pressel *et al.*, 2008; Jang & Dolan, 2011). The transition from chloronema to caulonema is normally gradual, such that intermediate cell types with increasing caulonema characteristics toward the apex are usually found along protonema filaments (Jang & Dolan, 2011). Therefore, when culture on solid media overlaid with cellophane (a cellulosic material that prevents the colony from growing into the media but allows diffusion of water and nutrients), a germinating spore or a small inocula of protonema tissues will typically develop into a protonema colony with a greener centre composed of more chloronema-like cells and a paler edge composed of more caulonema-like cells (**Figure 1-7**). Secondary protonema branches are formed from either caulonema and chloronema is always chloronemal when they first develop (**Figure 1-7)**. The main function of chloronema cells appears to be photosynthesis and assimilation whereas caulonema cells function to explore the surroundings and potentially transport of solute (Duckett *et al.*, 1998).

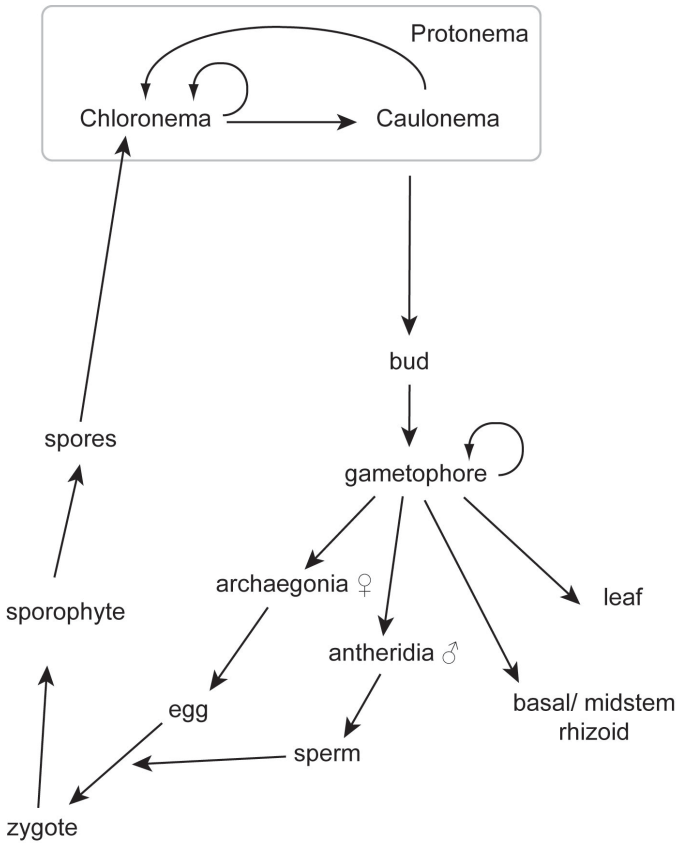


Figure 1-6 Life cycle of *P. patens*. For details see text.

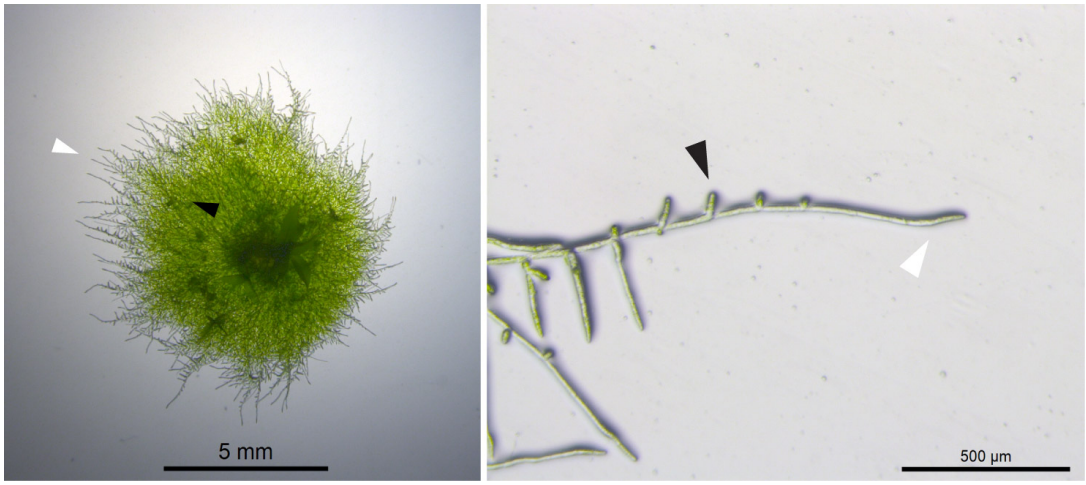


Figure 1-7 The protonema stage of *P. patens*. The protonema stage consists of two types of tip-growing filaments: the chloronema and the caulonema. Left: a protonema colony. The centre of the colony are composed of the greener chloronema filaments, which appear to function in photosynthesis and assimilation as they posses more chloroplasts. Chloronema filaments gradually develop into caulonema filaments as they grow away from the centre of the colony. Caulonema filaments contain less chloroplasts and appear to function in exploring the surroundings and potentially transport of solute. Right: close-up image of a protonema filament at the edge of a colony. Black arrowheads indicate chloronema filaments. White arrowheads indicate caulonema filaments. See text for more details.

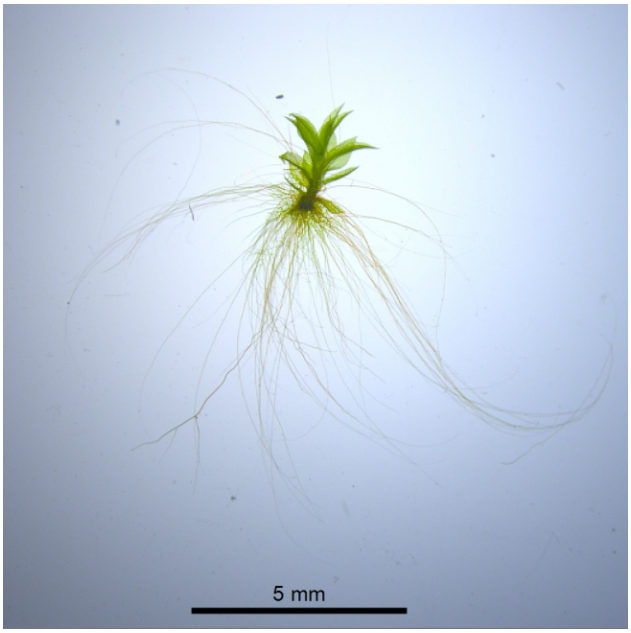


Figure 1-8 A mature *P. patens* gametophore. The gametophore develops from an apical cell found at the apex. Brown rhizoids develop near the base of the gametophore, and provide anchorage. See text for more details.

As the protonemal colony matures, bud initials that will give rise to the gametophore begin to develop from the protonema. The mature gametophore comprises a green leafy shoot-like axis with leaf-like appendages (**Figure 1-8**). Brown-pigmented rhizoids develop from the base (basal rhizoids) and also mid-stem (mid-stem rhizoids) (Sakakibara *et al.*, 2003; Jang *et al.*, 2011). *P. patens* rhizoids are cytologically similar to caulonema except that they are pigmented (Duckett *et al.*, 1998; Pressel *et al.*, 2008). It has also been suggested that the food-conducting cells in the gametophore are cytologically similar to rhizoids and caulonema (Ligrone *et al.*, 2000; Pressel *et al.*, 2008).

As the gametophore matures, sexual organs, the antheridia and the archegonia, are usually produced near the apex of the shoot. (Ashton & Raju, 2000; Landberg *et al.*, 2013). The apical cell, which normally gives rise to the metamer of leaf and side-branch initials, is consumed in the production of the archegonia (La Farge-England, 1996; Landberg *et al.*, 2013). After the apical cell is consumed, a side-branch initial begins to develop into a new branch near the old apex leading to a acrocarpous, sympodial growth form (La Farge-England, 1996). Basal rhizoids normally develop from the base of this new gametophore (**Figure 1-8**). Fertilization takes place when a sperm cell fertilizes an egg cell inside the archegonium (Landberg *et al.*, 2013). The embryo then develops into the sporophyte, which is dependent on the gametophyte. Each sporophyte contains around 4000 spores (Cove, 2005), which germinate to produce the next generation.

1.6.1 The effect of auxin on *P. patens* development

Auxin affects almost every stage of *P. patens* development. During the protonema stage, application of exogenous auxin strongly inhibits chloronema growth,

promotes caulonema development, increases pigmentation in both chloronema and caulonema, and inhibits gametophore bud formation (Ashton *et al.*, 1979; Ashton, 1998; Jang & Dolan, 2011). In the mature gametophore, auxin alters leaf and gametophore morphology, and positively regulates rhizoid development (Sakakibara *et al.*, 2003; Decker *et al.*, 2006; Eklund *et al.*, 2010a; Jang *et al.*, 2011). Auxin also inhibits gametophore side-branch formation in *Plagiomnium cuspidatum* (Bryales) *and Splachnum ampullaceum* (Funariales; same order as *P. patens*) (Von Maltzahn, 1959; Nyman & Cutter, 1981)*;* but whether this is also true for *P. patens* is not known.

The distribution of auxin in *P. patens* has been characterized by using the auxin-inducible GH3 promoter fused to the GUS reporter gene (Bierfreund *et al.*, 2003; Fujita *et al.*, 2008). GUS signal is strongest in the base of the gametophores, and in both basal and mid-stem rhizoids (Fujita *et al.*, 2008). The distribution of auxin in the protonema, however, is unclear (Bierfreund *et al.*, 2003).

Most of the auxin produced by *P. patens* appear to be secreted into the media (Reutter *et al.*, 1998; Schween *et al.*, 2003), suggesting that exogenous concentration of auxin may be important for auxin action. However, as in angiosperms (Teale *et al.*, 2006; Del Bianco & Kepinski, 2011), mechanisms such as localized auxin biosynthesis, cell-type specific rate of auxin uptake or sensitivity to auxin, and potential auxin transport mechanisms (at least in the protonema stage) are all important factors of auxin action. In the moss *Funaria hygrometrica,* which belongs to the same family as *P. patens* (Goffinet & Shaw, 2009), polar transport of auxin at the protonema stage has been shown to be important in caulonema differentiation (Johri & Desai, 1973; Rose *et al.*, 1983). Treatment of protonema

with p-chlorophenoxyisobutyric acid (PCIB), which blocks polar auxin transport in *F. hygrometrica* rhizoids (Rose & Bopp, 1983), resulted in the stimulation of secondary chloronema formation (exogenous auxin inhibits chloronema formation). In *P. patens*, treatment with the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) also suppresses rhizoid development in *P. patens* (Jang, 2011). In addition to transport, the uptake of auxin from the media may also contribute to auxin distribution in mosses: rate uptake of IAA is about two times higher at the base of a rhizoid filament than at the tip in *F. hygrometrica* (Rose & Bopp, 1983).

Local synthesis of auxin may be important in moss development. In Arabidopsis, the *SHORT-INTERNODES/STYLISH (SHI/STY)* family proteins are transcriptional activator of auxin biosynthesis genes such as members of the *YUCCA* family genes (Eklund *et al.*, 2010a). Their homologs in *P. patens*, *PpSHI1* and *PpSHI2,* are also positive regulators of auxin biosynthesis (Eklund *et al.*, 2010b). *PpSHI1* and *PpSHI2* are expressed most strongly in caulonema, rhizoids, and axillary hairs of the gametophores (Eklund *et al.*, 2010b). *Ppshi1* and *Ppshi2* mutants have decreased level of endogenous auxin and reduced caulonema development and gametophore intermodal length, whereas overexpression of the *PpSHI* genes resulted in increased endogenous auxin level and the stimulation of caulonema and suppression of caulonema and increased pigmentation of the caulonema (Eklund *et al.*, 2010b). However, rhizoid number is not affected in the *Ppshi* mutants, even though exogenous auxin promotes rhizoid number (Eklund *et al.*, 2010b). Interestingly, although *Ppshi1* and *Ppshi2* mutants are defective in gametophore internode elongation, *PpSHI* and *PpSHI2* seems not to be expressed in the intermodal region *per se*, but on axillary hairs, suggesting potential diffusion mechanisms from the axillary hairs to the elongating stem of the gametophores,

even though polar auxin transport may not operate in the moss gametophore (Fujita *et al.*, 2008).

The mechanism of auxin signalling is relatively well understood in *A. thaliana*. One major pathway involves auxin binding to the auxin receptor SCF^{TIR1} complex, (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005). This complex targets Aux/IAA proteins for ubiquitin-mediated degradation. Degradation of Aux/IAA proteins results in de-repression of Auxin response factors (ARFs). De-repressed ARFs can either activate (ARF^{A}) or repress (ARF^{R}) the expression of auxin-responsive genes (Teale *et al.*, 2006; Del Bianco & Kepinski, 2011).

Major auxin signalling components, including 4 TIR1/AFB auxin receptors, 2 (or 3) Aux/IAA proteins, and 14 ARFs are present in the genome of *P. patens* (Rensing *et al.*, 2008; Prigge *et al.*, 2010), suggesting that similar mechanisms may operate in *P. patens*. Nearly all of these auxin-signalling components are present in the genome of *P. patens* (Rensing *et al.*, 2008). In *P. patens*, knocking down the *TIR1* homologs *PpAFB1* to *PpAFB4* resulted in suppression of caulonema development (Prigge *et al.*, 2010). Consistent with the fact that the SCF^{TIR1} complex targets Aux/IAA protein for degradation, *P. patens* mutants that show defect degron motif in *Ppiaa* genes show phenotypes that are comparable to those of the *Ppafb* knockdown mutants (Prigge *et al.*, 2010). It has also been shown that exogenous auxin induces the expression of all three *PpIAA* genes (*PpIAA1A*, *PpIAA1B* and *PpIAA2*) and that *Ppiaa* mutants are insensitive to auxin (Prigge *et al.*, 2010). Together these results suggest that auxin signalling in the chloronema, which are necessary for the development of caulonema, are mediated through the *PpIAA* and *PpAFB* proteins. Additionally, mutations in the *P. patens* homolog of the tomato *DGT* gene, which

are likely to be involved in auxin perception or response, show defective chloronema-to-caulonema transition and rhizoid development (Lavy *et al.*, 2012). However, *PpIAA1B* degradation is not affected in the *Ppdgt* mutants, and that only a subset of auxin-response genes are affected, suggesting that *PpDGT* affects auxin perception or response in a specific pathway that may be Aux/IAA independent (Lavy *et al.*, 2012).

This effect of auxin on chloronema-to-caulonema transition is dependent on the Class I *RSL* genes *PpRSL1* and *PpRSL2* (Jang & Dolan, 2011). *Pprsl1 Pprsl2* mutants are resistant to auxin and do not develop more caulonema when treated with auxin, whereas overexpression of both *PpRSL1* and *PpRSL2* resulted in increased caulonema differentiation (Jang & Dolan, 2011). Together, these results suggested that auxin positively regulates the chloronema-to-caulonema transition via *PpRSL1* and *PpRSL2*. Rhizoid development is also positively regulated by auxin via the *PpRSL* genes. Exogenous auxin induces the development of both basal and mid-stem rhizoids (Ashton *et al.*, 1979; Sakakibara *et al.*, 2003). Auxin induce the expression of *PpRSL1* and PpRSL2 and fails to induce rhizoid development in the *Pprsl1 Pprsl2* double loss-of-function mutants; overexpression of *PpRSL1* and *PpRSL2* resulted in increased rhizoid development (Jang *et al.*, 2011). These results suggest that auxin induces rhizoid development via *PpRSL1* and *PpRSL2*. *PpRSL1* and *PpRSL2* also modulate the response of the Class II *RSL* genes (*PpRSL3, PpRSL4, PpRSL5, PpRSL6*) to auxin (Pires *et al.*, 2013).

Pigmentation is an indication of differentiation into the rhizoid cell fate and is one of the features that distinguish rhizoids from caulonema. Once rhizoid cell fate has been determined, the HD-ZIP gene *PpHB7*, which controls pigmentation of the

rhizoid, starts to be expressed in the rhizoids; *PpHB7* is not expressed in chloronema or caulonema (Sakakibara *et al.*, 2003). The transcript level of *PpHB7* is increased by exogenous auxin, and mutants with a defective *PpHB7* gene resulted in defective pigmentation, and the rhizoids are greener than wild-type (Sakakibara *et al.*, 2003). However, number and position of the rhizoids are not affected in the *Pphb7* disruptant lines (Sakakibara *et al.*, 2003). These results suggest that auxin controls rhizoid differentiation through the *PpHB7* gene.

1.6.2 The cytoskeleton plays important role in protonema and rhizoid development

As is common in tip-growing cells like pollen tubes and root hairs, the cytoskeleton – including actin microfilaments and microtubules – plays an important role in protonema development in *P. patens*. (Doonan *et al.*, 1988; Vidali & Bezanilla, 2012; Rounds & Bezanilla, 2013). Although microtubules have been shown to be active during protonema development in *P. patens* (Doonan *et al.*, 1988), actin appears to be more important and consequently its role is better characterized.

A dynamic actin cytoskeleton is essential for tip growth, and this involves dynamic balance between actin polymerization and depolymerization, and the organization of the actin filaments in bundles in the cell (Vidali *et al.*, 2007). For example, treatment with Cytochalasin D, which inhibits actin polymerization led to arrested tip growth in the protonema (Doonan *et al.*, 1988).

The dynamic remodeling of actin microfilaments is regulated by a number of actin binding proteins, which regulates the active polymerization and depolymerization of actin microfilaments. *P. patens ACTIN INTERACTING PROTEIN1 (PpAIP1)* and ACTIN DEPOLYMERIZING FACTOR (*PpADF)* regulate actin depolymerization.

Loss-of-function mutation in the *PpAIP1* gene led to defective actin depolymerization, resulting in stunted growth in chloronema and rhizoids and the absence of caulonema cells (Augustine *et al.*, 2011). This defect can be partially complemented by overexpressing *PpADF* in plants that lack *PpAIP1* gene function, indicating that PpAIP1 protein acts partly through PpADF to control actin dynamic turnover, and in turn regulate rhizoid and protonema growth (Augustine *et al.*, 2011).

Several groups of protein regulate actin polymerization. Profilins recharge monomeric ADP-actin with ATP and direct their assembly into growing actin filaments; loss-of-function mutants of *P. patens* profilin genes led to disorganized actin filaments and the abolition of tip growth in the protonema (Vidali *et al.*, 2007). Class II formins are also essential for tip-growth in *P. patens*: loss-of-function mutations in class II formins in *P. patens* abolishes tip growth and led to plants composing of spherical cells (Vidali *et al.*, 2009). Similarly, mutations in genes encoding the subunits of the *P. patens* Arp2/3 complex (which regulates actin polymerization), including *PpARPC1*, *PpARPC3*, and *PpARPC4*, led to defective chloronema growth and severely stunted rhizoids or caulonema development (Harries *et al.*, 2005; Perroud & Quatrano, 2006; Finka *et al.*, 2008). *PpPIPK1* and *PpPIPK2*, which encode enzymes that control the synthesis of phosphoinositides, also control caulonema and rhizoid development via the actin cytoskeleton (Saavedra et al., 2009; Saavedra et al., 2011). Wild-type plants treated with cytochalasin B, a drug that prevents actin polymerization, resembles the *Pppipk1* mutants without drug treatment, which shows a disorganized filamentous actin network at the tip of caulonema cells (Saavedra *et al.*, 2011). Loss-of-function *Pppipk1* mutants resulted in impaired caulonema and rhizoid development, and the

Ppppipk1 Pppipk2 double loss-of-function mutants resulted in no caulonema and shorter rhizoids than the *Pppipk1* mutants (Saavedra *et al.*, 2011). Interestingly, Loss-of-function mutations in *PpARPC1*, *PpARPC3*, *PpARPC4*, and the *PpPIPKs*, also affect gametophore development (Harries *et al.*, 2005; Perroud & Quatrano, 2006; Finka *et al.*, 2008; Saavedra *et al.*, 2011), but whether this is a secondary effect due to defective caulonema and rhizoid is unclear.

In *A. thaliana* root hair, Myosin XIs contribute to cytoplasmic streaming and also filamentous actin organization (Prokhnevsky *et al.*, 2008; Peremyslov *et al.*, 2010). Although organelles movements in *P. paten* protonema are slow compared to that of *A. thaliana* root hair, indicating that rapid cytoplasmic streaming may not be in operation in *P. patens* protonema, moss homologs of myosin XIs are nevertheless essential for tip growth (Vidali *et al.*, 2010). Loss-of-function mutations in both of the two myosin XI genes in *P. patens* resulted in the abolishment of tip growth and the formation of spherical cells (Vidali *et al.*, 2010). This may be explained by the role myosin XIs play in organizing the actin cytoskeleton in the tip of the protonema (Furt *et al.*, 2012).

Another class of myosin, myosin VIII, has also been shown to be important for *P. patens* development, although their interaction with the cytoskeleton is less clear. *P. patens* plants with myosin VIII genes knocked down by RNAi (Δ*myo8ABCDE*) show increased protonema branching, curved protonemal filaments and early gametophore formation (Wu *et al.*, 2011). Interestingly, application of exogenous auxin partially rescues the protonemal defects in these mutants (Wu *et al.*, 2011). This suggests that Myosin VIII may regulate auxin homeostasis or transport in *P. patens* protonema.

1.6.3 Low phosphate induces caulonema and rhizoid development in *P. patens*

Protonema and rhizoid development in *P. patens* is also affected by environmental stimuli such as phosphate availability. In *A. thaliana*, the *PHO1* family genes are involved in phosphate loading into xylem and phosphate homeostasis; and *AtPHO1* is up regulated during phosphate deficiency (Wang *et al.*, 2004; Stefanovic *et al.*, 2007). Low phosphate stimulate caulonema and rhizoid development and increase the expression of *PpPHO1;1* and *PpPHO1;7* (Wang *et al.*, 2008). These results indicate that the response to low phosphate by the rooting systems, and the function of *PHO1* family genes may be conserved in land plants.

1.6.4 *P. patens* development is affected by other factors

In addition to the factors mentioned above, wavelength of light and its intensity (Imaizumi, 2002), carbon source and energy supply (Olsson *et al.*, 2003; Thelander *et al.*, 2005) all play a role in moss development and in particular the chloronema to caulonema transition. The hormones strigolactone, cytokinin (which often act antagonistically to auxin) and abscisic acid (ABA) also control *P. patens* development (Ashton *et al.*, 1979; Decker *et al.*, 2006; Proust *et al.*, 2011).

2 *LRL* genes are positive regulators of rhizoid and caulonema development in *P. patens*

2.1 Introduction

The *LRL* (*Lotus japonicus RHL1-LIKE)* genes are an ancient group of bHLH transcription factors that control root hair development in diverse angiosperms including the monocot *Oryza sativa*, and the eudicots *Lotus japonicus* and *Arabidopsis thaliana* (Ding *et al.*, 2009; Karas *et al.*, 2009; Bruex *et al.*, 2012). In *A. thaliana*, *AtLRL3* is transcriptionally regulated by Class I *RSL* genes, which are positive regulators of root hair development (Karas et al., 2009). These led to the hypothesis that the *LRL* genes may be ancient, conserved regulators of the development of tip-growing rooting cell in the common ancestor of land plants. To test this hypothesis, I characterized the *LRL* genes in the moss *P. patens* by determining their expression pattern and characterizing the phenotypes of loss-of-function and gain-of-function mutants.

2.2 Materials and methods

2.2.1 Plant material and Growth conditions for *P. patens*

The Gransden wild-type strain (Ashton & Cove, 1977) of *Physcomitrella patens* (Hedw.) Bruch & Schimp was used in this study. Unless otherwise stated, all plants are grown in a SANYO MLR-351 growth chamber at 25˚C and a 16 h light / 8 h dark regime with a photosynthesis photon flux density of approximately 40 mol m^{-2} s^{-1}.

Plants are grown on a modified KNOPS medium (“The minimal media” or “KNOPS-GT”) is used (Ashton & Cove, 1977): 0.8g/L $CaN_2O_6 \cdot 4H_2O$, 0.25g/L $MgSO_4 \cdot 7H_2O$, 0.0125g/L $FeSO_4 \cdot 7H_2O$, 0.055mg/L $CuSO_4 \cdot 5H_2O$, 0.055mg/L

$ZnSO_4 \cdot 7H_2O$, 0.614mg/L H_3BO_3, 0.389mg/L $MnCl_2 \cdot 4H_2O$, 0.055mg/L $CoCl_2 \cdot 6H_2O$, 0.028mg/L KI, 0.025mg/l $Na_2MoO4 \cdot 2H_2O$, 25mg/L KH_2PO_4 buffer (i.e. 1.84mM at pH 6.5 with KOH) and 7g/L agar (Formedium AGA03). 5g/L glucose and 0.5g/L ammonium tartrate dibasic ($C_4H_6O_6 \cdot 2H_3N$) was added as supplements for routine subculture of macerated protonema tissues ("the KNOPS" media). Sterile cellophane discs (80 mm 325P Cellulose discs; A.A. Packaging Ltd.) are put on top of the media to facilitate phenotypic analysis and collection of tissues for subculture. 0.1% dimethyl sulfoxide (DMSO) is sometimes added to the media as a control for auxin treatment (which involves adding auxin dissolved in DMSO).

To generate spores, macerated protonema tissues were dispense onto sterile, irrigated 44mm Jiffy 7 peat pellets contained in Magenta GA-7 vessels (Sigma, UK) and were first cultured for about 1 month in a SANYO MLR-351 growth chamber at 25˚C and a 16 h light / 8 h dark regime, before being transferred to a 16˚C, 8h light / 16h dark regime (with same light intensity) to induce spore formation. Mature, intact sporangia were harvested and surface-sterilized in 0.5% (v/v) sodium hypochlorite for 5 mins and rinsed at least 4 times with sterile water. Spores are released into suspension in water by gently crushing the submerged sporangia with a pipette tip.

2.2.2 Sequence retrieval and alignment

Sequences were retrieved from BLAST searches with *AtLRL3 (At5g58010)* as a query on Phytozome v9.1 (Goodstein *et al.*, 2011), the *Lotus japonicus* genome assembly build 2.5 (http://www.kazusa.or.jp/lotus/index.html), and the Amborella genome database (http://www.amborella.org/). Sequences from *Marchantia polymorpha* were obtained from unpublished data from the Liam Dolan lab

(Department of Plant Sciences, University of Oxford, UK). Where alternative transcripts exist, only the primary transcript was used. Coding sequences (CDS) retrieved from BLAST searches were first translated into amino acids in Seaview 4.4.1 (Gouy *et al.*, 2010) then aligned using Mafft with the L-INS-i option (Katoh & Standley, 2013). The alignments were then trimmed with TrimAI v1.3 with the automated1 method (Capella-Gutiérrez *et al.*, 2009) implemented in Phylemon 2.0 (Sánchez *et al.*, 2011) such that only unambiguously aligned columns are retained. The final amino acid alignment, containing 167 sequences from 33 species and 115 columns, was used in subsequent phylogenetic analyses and is given in **Appendix A1**.

2.2.3 Phylogenetic analyses

The best-fit model of amino acid evolution for the alignment was determined by Prottest 3 (Darriba *et al.*, 2011) and the JTT+G model was chosen for all alignments. The Maximum Likelihood tree was calculated with PhyML 3.0 (Guindon *et al.*, 2010) using a JTT+G model with aLRT and the best of NNI (Nearest Neighbour Interchange) and SPR (Subtree Pruning and Regrafting) for tree improvement. Bayesian analysis was carried out with MrBayes v3.2.1 (Ronquist *et al.*, 2012) with the JTT+G model; two independent runs were computed until the average standard deviation of split frequency was less than 0.01. One tree was saved every 100 generations and the first 25% of trees were discarded. A 50-Majority-Rule tree was generated from the remaining trees. Trees were visualized with Figtree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/)

2.2.4 Generation of *P. patens* complete knockout mutants and GUS-reporter lines

Three backbone plasmids were used for generating the knock-out and GUS-reporter constructs used in this study: pBHrev (= pBHSNR) and pBNRF (Menand *et al.*, 2007b), and pBHrev-GUS (= pBHSNR-GUS) (Jang *et al.*, 2011). To generate the *Pplrl1* knockout mutants, an 812bp DNA fragment upstream and a 819bp fragment downstream of the *PpLRL1* (Pp1s173_83V6; gene annotation v1.6) were cloned into the SalI-BamHI and MluI-NcoI sites of the pBHrev plasmid respectively. Similarly, to generate *Pplrl2* knockout mutants, a 697bp fragment upstream and a 655 fragment downstream of the *PpLRL2* locus (Pp1s209_22V6; gene annotation v1.6) were cloned into the SalI-BamHI and SpeI-AscI site of the pBNRF plasmid respectively. These generated the pBHrev-PpLRL1KO and pBNRF-PpLRL2KO construct. The plasmids were linearized with SalI + NcoI (for pBHreb-PpLRL1KO) and SalI + AscI (for pBNRF-PpLRL2KO) before PEG-mediated transformation. Stable transformants were verified by PCR and Southern blot (**Figure 2-1).** Three independent lines that show only a single insertion at the target locus are kept for each genotype.

To generate the GUS reporter lines for *PpLRL1*, a 871bp fragment upstream and a 888bp fragment downstream of the predicted coding sequence were cloned into the AscI-NcoI and BamHI site, in the correct orientation, of the pBHrev-GUS plasmid, respectively. Similarly, for the GUS-reporter lines for *PpLRL2*, a 903bp fragment upstream and a 880bp fragment downstream of the predicted coding sequence were cloned into the AscI-NcoI and BamHI-HindIII site of the pBHrev-GUs plasmid, respectively. These generated the pBHrev-PpLRL1proGUS and pBHrev-PpLRL2proGUS constructs. The plasmids were linearized with AscI (for pBHrev-

PpLRL1proGUS) and AscI + HindIII (for pBHrev-PpLRL2proGUS) before PEG-mediated transformation. Three independent lines that contained a single insertion at the target locus, as verified by PCR and Southern blot were kept for each genotype (**Figure 2-2**). Southern blots were carried out with the DIG system for filter hybridization with DIG-labelled probe generated by PCR (Roche). Primers for probe synthesis are given in **Appendix A5.**

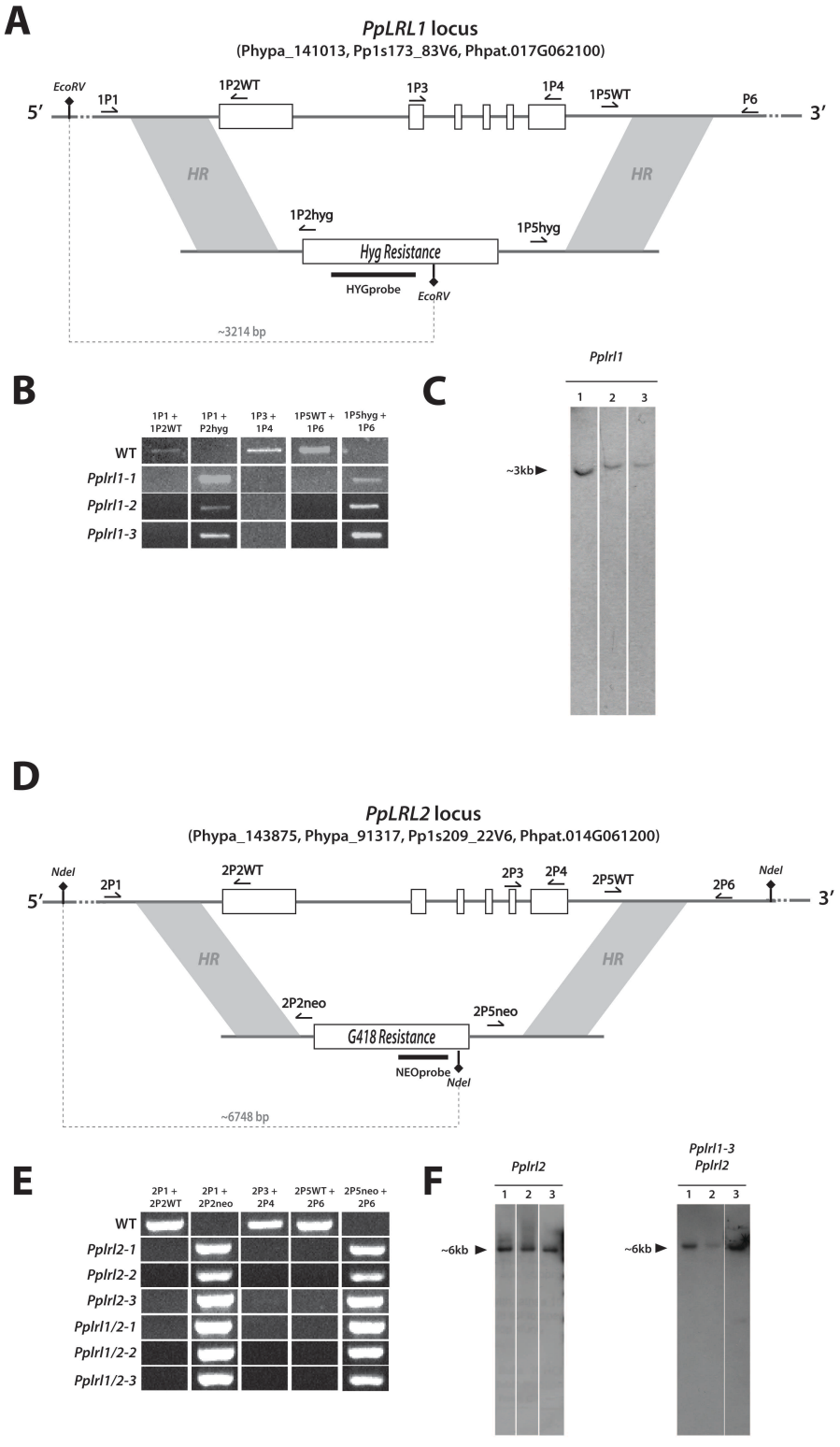


Figure 2-1 Verification of *Pplrl1*, *Pplrl2*, and *Pplrl1 Pplrl2* mutants. A,D) Schematic of gene targeting by homologous recombination; B,E) PCR Verification of mutants; C,F) Southern blot analysis showing a single insertion in each line.

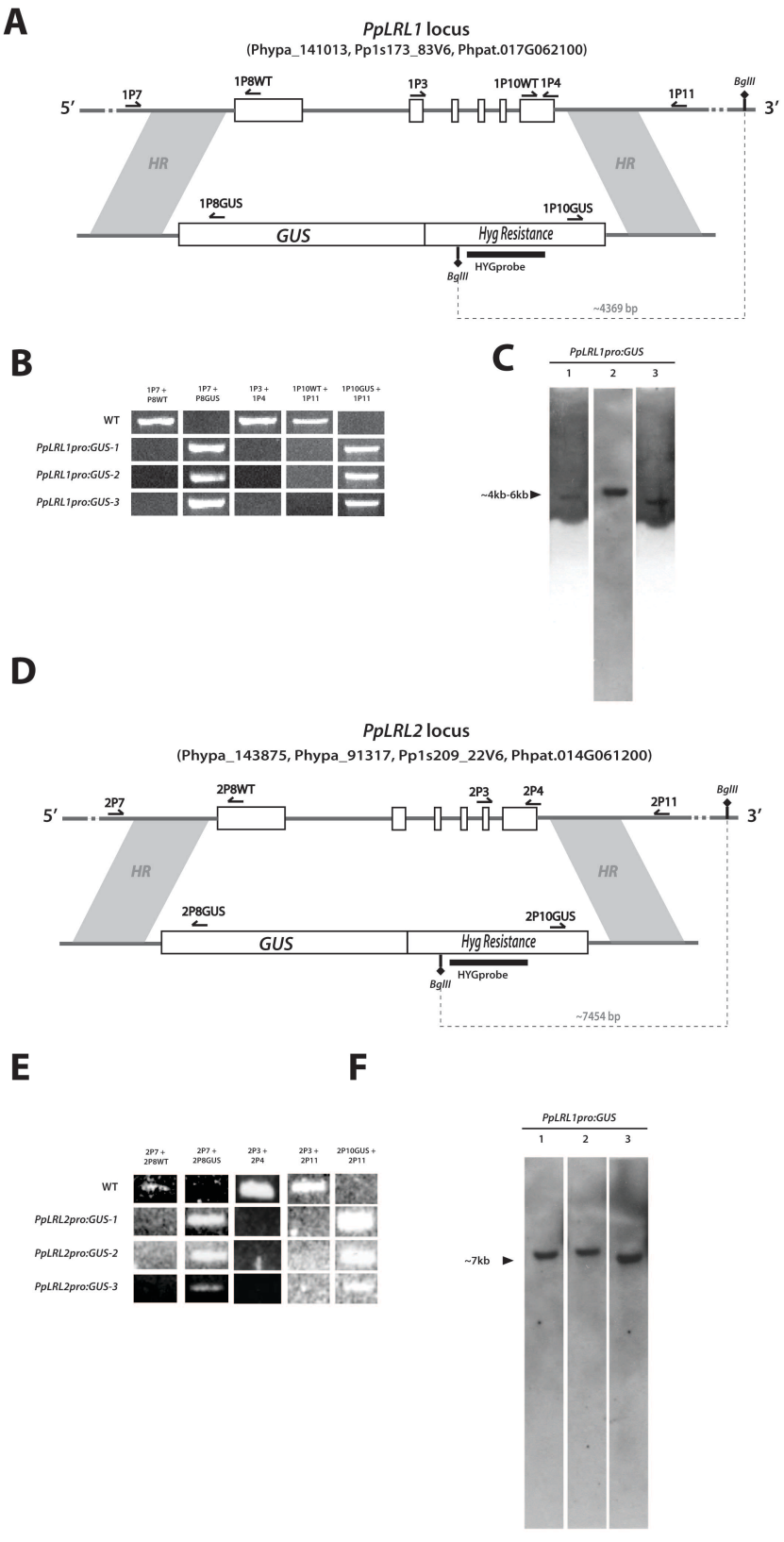


Figure 2-2 Verification of *PpLRL1pro:GUS* and *PpLRL2pro:GUS* reporter lines. A,D) Schematic of gene targeting by homologous recombination; B,E) PCR Verification of mutants; C,F) Southern blot analysis showing a single insertion in each line.

2.2.5 Generation of *P. patens* partial gene deletion mutants and partial over-expression mutants

Partial gene deletion mutants (*Pplrl1*Δ*bHLH-LRL* and *Pplrl2*Δ*bHLH-LRL*) where only the bHLH and LRL domains are deleted were generated similarly (**Figure 2-11)** and were PCR verified. Two independent lines of each genotype were generated and they show the same phenotypes. To generate the *35S:PpLRL-bL* and *35S:PpLRL2-bL* mutants, partial coding sequences for the bHLH-LRL region of the *PpLRL* genes **(Figure 2-11)** were first amplified by RT-PCR and subcloned into the pCR8/GW/TOPO Vector (Invitrogen) then transferred to the p108GW35S vector through the Gateway LR Clones II system (Invitrogen) for moss over-expression (Pires *et al.*, 2013). This construct includes the 108 locus to facilitate insertion into the neutral 108 locus in the *P. patens* genome. The steady-state transcript levels of *PpLRL1*-bL and *PpLRL2-bL* transcripts in selected *35S:PpLRL-bL* lines were determined by qRT-PCR (**Figure 2-3**).

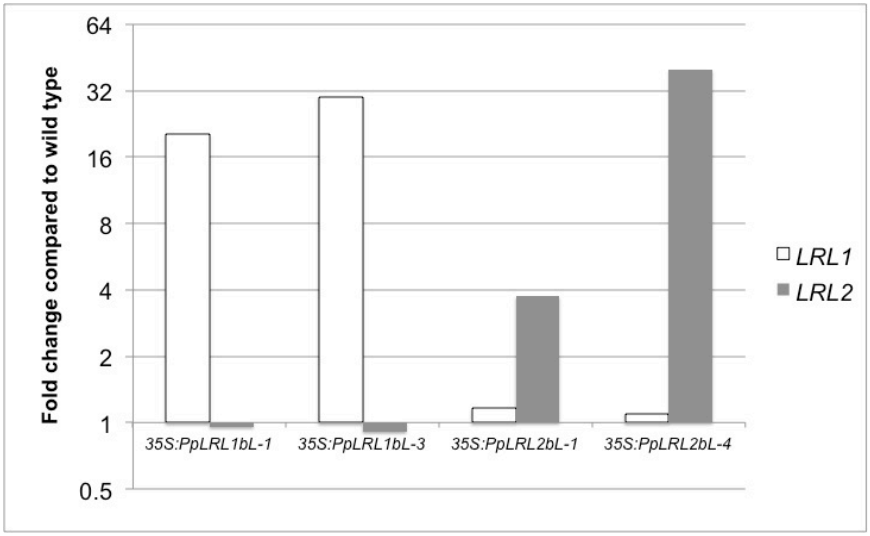


Figure 2-3 Expression level *PpLRL-bL* transcript in selected 35S:*PpLRL*bL lines using Ade PRT (Phypa_443007) and E2 (Phypa_430768) as reference genes (Le Bail *et al.*, 2013) and PpLRL_q3F/R as qPCR primers (Appendix A5).

2.2.6 PEG-mediated transformation of P. patens

PEG-mediated transformation was performed as described previously (Schaefer & Zrÿd, 1997; Menand *et al.*, 2007b). Antibiotic selection was done with 50µg/ml G418 disulfate or 25µg/ml hygromycin B.

2.2.7 qRT-PCR

Total RNA was extracted from frozen protonema tissue with the RNeasy Plant Mini Kit (Qiagen). The RNA was then quantified with a NanoDrop ND1000 spectrometer (Thermo Scientific) and the same amount of RNA from all samples was treated with Turbo DNAse (Ambion, Life Technologies) and used for first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies) using oligo(dT). The DNAse-treated total RNA was used for qRT-PCR analysis with SYBR Green PCR Master Mix (Applied Biosystems) and the Applied Biosystems 7300 Real Time PCR system. Three technical replicates were used for each sample. The cycling conditions were as follows: 50˚C 2 min; 95˚C 10 min; then 40 cycles of 95˚C 15 sec, 60˚C 1 min. Dissociation curve analyses were performed at the end of the cycles to ensure the formation of only a single amplicon for each replicate. Baseline and Ct threshold was determined automatically using the bundled software with the Real Time PCR machine. Unless otherwise stated, *PpGAPDH* (Pp1s353_25V6) and *PpEF1α* (Pp1s7_457V6) were used as reference genes and PpLRL1_CDS_ primers (**Appendix A5**) were used to determine transcript levels of *PpLRL* genes. Fold change was calculated with the delta-delta-Ct method in Microsoft Excel using *PpGAPDH* as the internal reference. The list of primers used is given in the **Appendix A5**.

2.2.8 GUS Staining

Unless otherwise stated, protonema inocula were grown on KNOPS media with cellophane for 2.5 weeks and then directly incubated in GUS staining solution: 100mM NaH_2PO_4 (pH 7.0 with NaOH), 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6 \cdot 3H_2O$, 0.05% Triton X-100, 1mM 5-Bromo-4-chloro-3-indolyl-ß-D-glucuronic acid, cyclohexyl ammonium salt (X-GlcA; Melford, UK) dissolved in N,N-Dimethylformamide (DMF) at 37˚C for 24-48 hours. No staining was observed in wild type plants under any condition. Tissues were then de-stained by incubation in 70%-100% ethanol for a total of at least 24 hours.

2.2.9 Time lapse microscopy and image analysis

Protonema filaments at the edge of a protonema colony growing on cellophane discs on minimal media were photographed every 6 mins under a Leica DFC310 FX camera mounted on a Leica M165 FC stereomicroscope. Images were analyzed with ImageJ (Schneider *et al.*, 2012).

2.3 Results

2.3.1 Two *LRL* genes are present in *P. patens*

LRL genes comprise subfamily XI of bHLH transcription factors that are characterized by the presence of a highly conserved "LRL" domain at the C-terminus end of the bHLH domain (Heim *et al.*, 2003; Pires & Dolan, 2010). Members of this subfamily perform many functions in plants, including controlling root hair development (Ding *et al.*, 2009; Karas *et al.*, 2009; Bruex *et al.*, 2012), hypocotyl and female gametophyte development in *Arabidopsis thaliana* (Pagnussat *et al.*, 2005; Hervé *et al.*, 2009), and phosphate starvation response in *Oryza sativa (Yi et al., 2005)*. Previous phylogenetic analyses recognize the monophyly of this subfamily, but the relationship among its members has been unclear to date (Buck & Atchley, 2003; Heim *et al.*, 2003; Toledo-Ortiz *et al.*, 2003; Li *et al.*, 2006; Carretero-Paulet *et al.*, 2010; Pires & Dolan, 2010). To resolve the relationships within land plant LRL proteins, I constructed a phylogenetic tree using an alignment of 167 LRL amino acid sequences from 33 taxa including *A. thaliana* and *P. patens* (**Figure 2-4, Appendix A1, A2)**. This provided a better resolution than previous studies. No *LRL* genes can be found in chlorophyte green algae including *Chlamydomonas reinhardtii*, suggesting that *LRL* genes originated in the streptophytes clade.

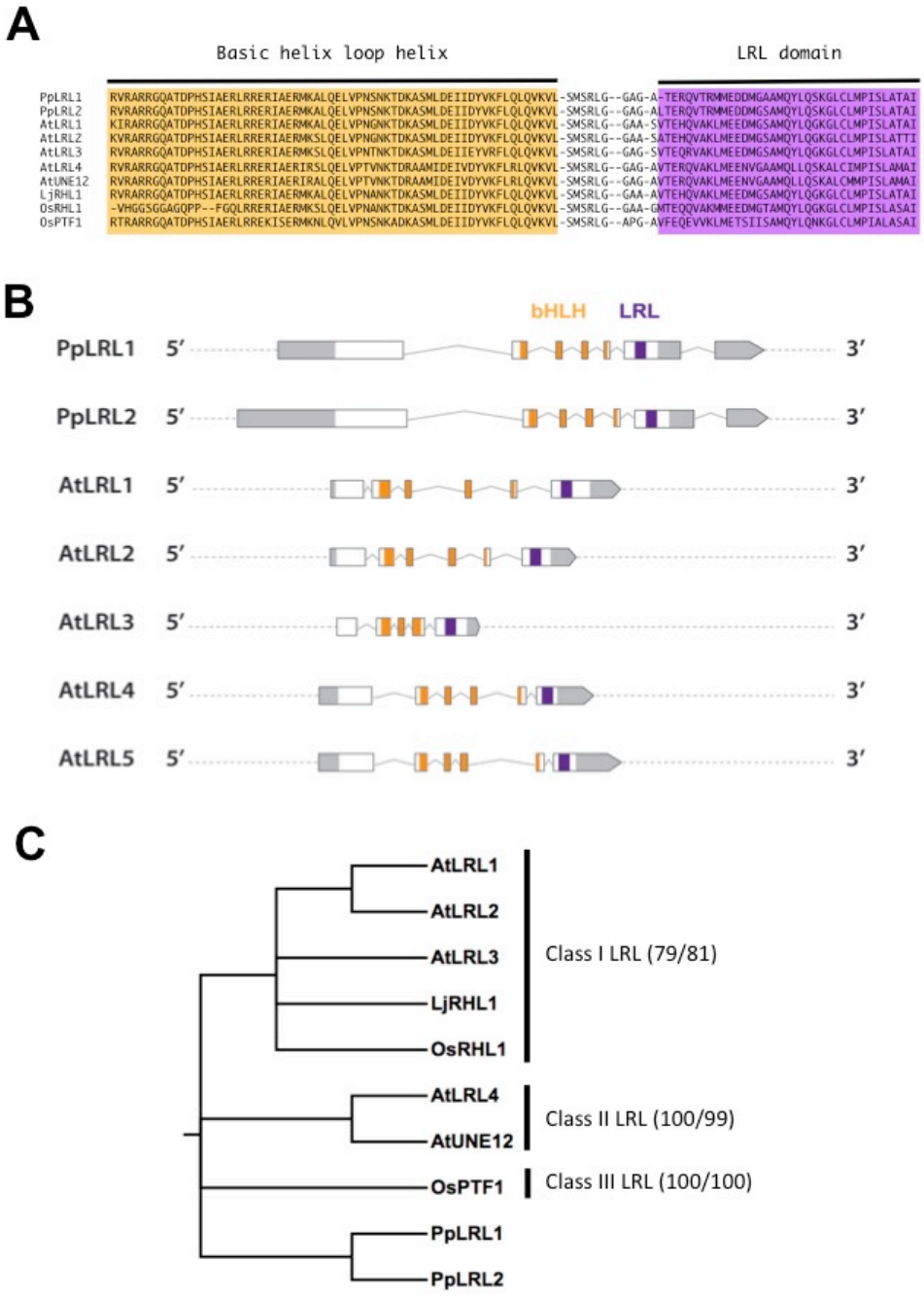
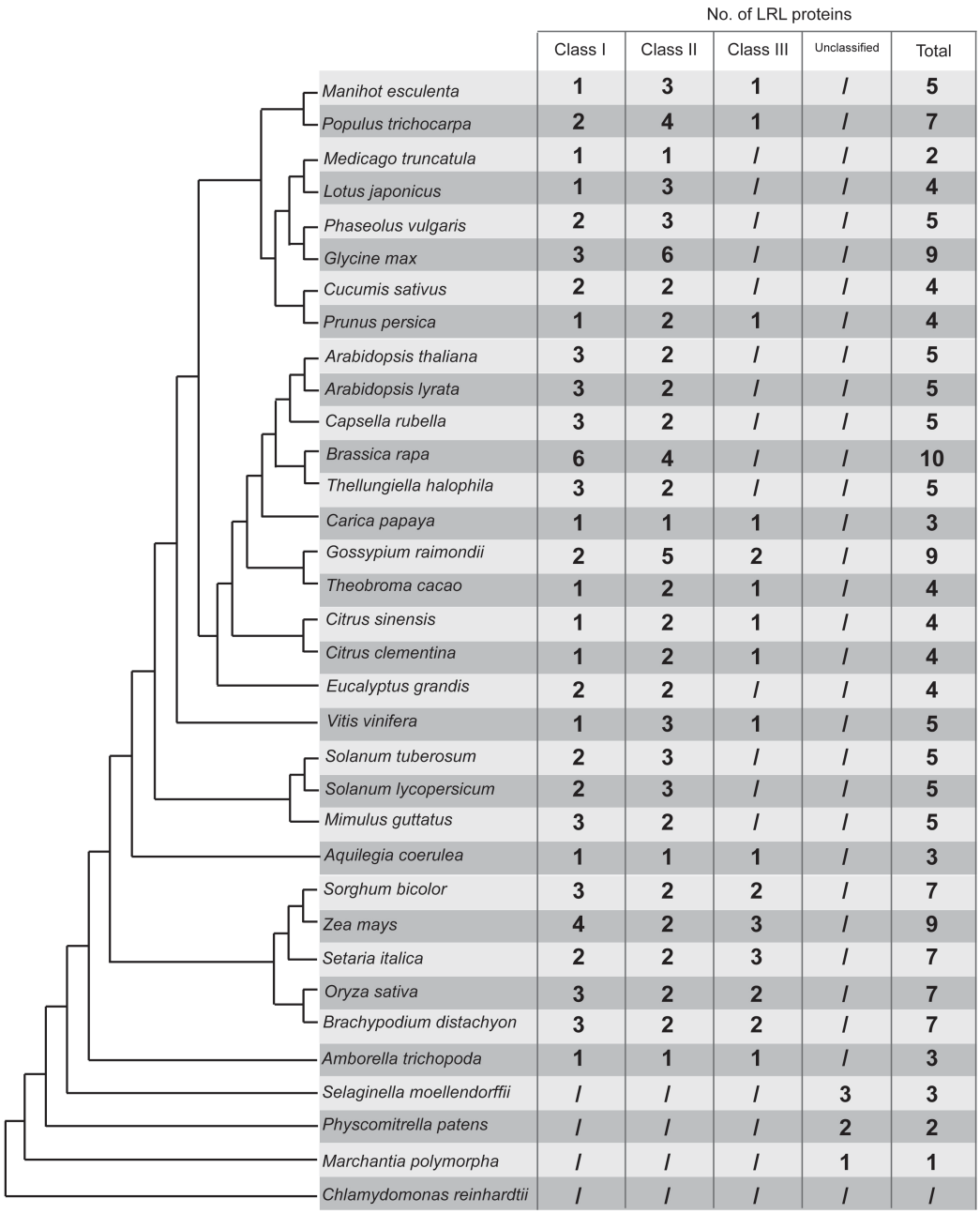


Figure 2-4 A: Part of the alignment used for phylogenetic analysis. The full alignment is given in Appendix A1. The bHLH and the LRL domain are highlighted. B: Gene model of selected *LRL* genes with the conserved bHLH and LRL domain highlighted. C: Phylogeny of the LRL proteins summarized from Bayesian and Maximum Likelihood analyses. The three classes of LRL indicated represent strongly supported clade in the full trees. Values in brackets indicate the Posterior probability and the aLRT values (in percentage) from the Bayesian and ML analyses, respectively).

Both Maximum likelihood and Bayesian analysis showed that land plant LRL proteins could be classified into three classes (**Figure 2-4. Appendix A2**). All the *LRL* genes that have been shown to regulate root hair development belong to Class I, including *AtLRL1 (bHLH66/ At2g24260)*, *AtLRL2 (bHLH69/ At4g30980)* and *AtLRL3 (bHLH82/ At5g58010)* in *A. thaliana* (Karas et al., 2009; Bruex et al., 2012), *LjRHL1* in *L. japonicus* (Karas et al., 2009) and *OsRHL1* (*Os06g08500)* in *Oryza sativa* (Ding *et al.*, 2009). Class II includes *AtLRL5 (AtUNE12 / bHLH59 / At4g02590)*, which has been implicated in hypocotyl and female gametophyte development (Pagnussat *et al.*, 2005; Hervé *et al.*, 2009), and *AtLRL4 (bHLH7/ At1g03040)*, which has not been characterized. Class III *LRL* genes are not found in *A. thaliana* and have also been lost multiple times in many eudicots lineages, but includes *OsPTF1 (Os06g09370)* that is involved in phosphate deficiency stress response in rice (Yi *et al.*, 2005).

The fact that the basal angiosperm *Amborella trichopoda* contains at least one *LRL* gene from each of the three classes suggests that this diversification of *LRL genes* into three distinct classes had already occurred in the last common ancestor of the angiosperms (**Figure 2-5**, see also the full trees in **Appendix A2**). However, my results indicate that this diversification had not occurred in the lycophyte *Selaginella moelendorffii*, the moss *Physcomitrella* patens, or the liverwort *Marchantia polymorpha.* Although *LRL* genes are present in these plants (*P. patens*, for example, have two closely related *LRL* genes; **Figure 2-4, Figure 2-5**), they do not belong to any of the three classes. This suggests that *LRL* genes in Bryophytes and lycophytes may represent an ancestral form of *LRL* genes.



	No. of LRL proteins				
	Class I	Class II	Class III	Unclassified	Total
Manihot esculenta	1	3	1	/	5
Populus trichocarpa	2	4	1	/	7
Medicago truncatula	1	1	/	/	2
Lotus japonicus	1	3	/	/	4
Phaseolus vulgaris	2	3	/	/	5
Glycine max	3	6	/	/	9
Cucumis sativus	2	2	/	/	4
Prunus persica	1	2	1	/	4
Arabidopsis thaliana	3	2	/	/	5
Arabidopsis lyrata	3	2	/	/	5
Capsella rubella	3	2	/	/	5
Brassica rapa	6	4	/	/	10
Thellungiella halophila	3	2	/	/	5
Carica papaya	1	1	1	/	3
Gossypium raimondii	2	5	2	/	9
Theobroma cacao	1	2	1	/	4
Citrus sinensis	1	2	1	/	4
Citrus clementina	1	2	1	/	4
Eucalyptus grandis	2	2	/	/	4
Vitis vinifera	1	3	1	/	5
Solanum tuberosum	2	3	/	/	5
Solanum lycopersicum	2	3	/	/	5
Mimulus guttatus	3	2	/	/	5
Aquilegia coerulea	1	1	1	/	3
Sorghum bicolor	3	2	2	/	7
Zea mays	4	2	3	/	9
Setaria italica	2	2	3	/	7
Oryza sativa	3	2	2	/	7
Brachypodium distachyon	3	2	2	/	7
Amborella trichopoda	1	1	1	/	3
Selaginella moellendorffii	/	/	/	3	3
Physcomitrella patens	/	/	/	2	2
Marchantia polymorpha	/	/	/	1	1
Chlamydomonas reinhardtii	/	/	/	/	/

Figure 2-5 Evolution of LRL protein in land plants. The LRL proteins diversified in the angiosperms into three distinct classes. See text for more details.

2.3.2 *PpLRL1* and *PpLRL2* show distinct expression patterns in *P. patens* gametophore

In *P. patens*, the rhizoid-bearing leafy shoots (the gametophores) begin their development with a defined sequence of cell division from the protonema, leading to the formation of a bud that includes a rhizoid precursor cell (Harrison *et al.*, 2009). As the bud develops into fully formed gametophores, numerous basal rhizoids develop from the base of gametophores (Sakakibara *et al.*, 2003; Jang *et al.*, 2011). As the gametophore matures, a few rhizoids also develop mid-stem (mid-stem rhizoids) directly under an adult leaf (Sakakibara *et al.*, 2003).

To determine whether *PpLRL* genes are expressed in rhizoids or their precursors, I generated GUS reporter lines by replacing the endogenous *PpLRL* genes with a GUS gene, generating the *PpLRL1pro:GUS* and *PpLRL2pro:GUS* lines, and determined GUS activity in gametophores at different stages of development (**Figure 2-6**). If *PpLRL* genes control rhizoid development, one might expect that they will be expressed in rhizoids. However, I detect no GUS signal in rhizoids in either *PpLRL1pro:GUS* or *PpLRL2pro:GUS* lines (**Figure 2-6**), suggesting that *PpLRL* genes are not strongly expressed in the rhizoids. Instead, GUS staining signal in for *PpLRL1* is strongest in apex and base of the gametophore, whereas *PpLRL2* appears to be most strongly expressed in the middle section of the gametophores (**Figure 2-6**). Since basal rhizoids initiate early at the base of the gametophore, the expression pattern of *PpLRL1* suggest that *PpLRL1* may control the initiation of rhizoid development, but not its maintenance. On the other hand, *PpLRL2* is not expressed at the base, but show stronger expression in the leaves. This suggests that *PpLRL2* may be involved in other aspects of *P. patens* development. The fact that GUS staining signal was observed in the early buds of

PpLRL1pro: GUS but not in *PpLRL2pro: GUS* lines also indicates that *PpLRL1* may act earlier than *PpLRL2*.

Taken together, these data suggest that *PpLRL1* may be involved in rhizoid development but their expression stops once rhizoids have developed, as in base of mature gametophores. There is also a possibility that *PpLRL* genes control gametophore and leaf development. Sectioning of stained *PpLRL1pro:GUS* and *PpLRL2proLGUS* should further define the exact cellular location of *PpLRL* expression in the gametophore.

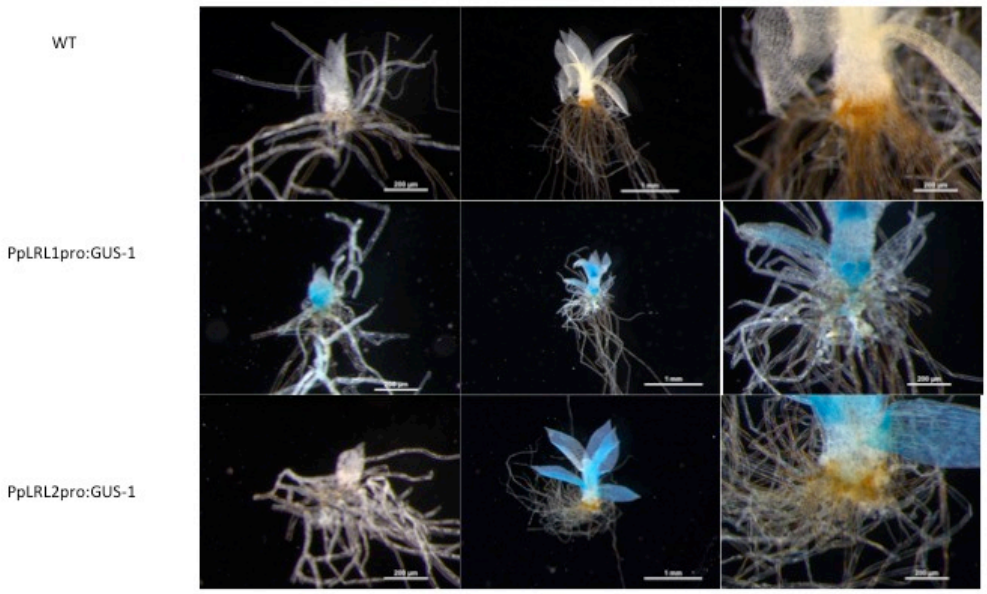


Figure 2-6 Expression pattern of *PpLRL* genes in developing buds and young gametophores. The first column show a young development bud, the second and third column shows a more mature gametophore. Note that GUS activity is detected in the early bud in the *PpLRL1pro:GUS* lines but not in the *PpLRL2pro:GUS* lines.

2.3.3 Both *PpLRL1* and *PpLRL2* are expressed in chloronema cells

In *P. patens*, there are three types of multicellular, tip-growing filaments: rhizoids, caulonema, and chloronema (Duckett *et al.*, 1998; Menand *et al.*, 2007a). Chloronema is the first cell type that develops when a spore germinates. As the colony develops, the chloronema filaments gradually differentiate into caulonema filaments. Chloronema filaments are chloroplast-rich and have a growth rate of about 6 μm h^{-1}; caulonema filaments are chloroplast-poor and have a growth rate of about 20 μm h^{-1} (Cove, 2005; Menand *et al.*, 2007a; Jang & Dolan, 2011). Caulonema filaments and rhizoids are morphologically similar (Duckett *et al.*, 1998; Pressel *et al.*, 2008), are affected in similar ways by low phosphate (Wang *et al.*, 2008) and are controlled by similar genetic components, such as *PpRSL1* and *PpRSL2* (Menand *et al.*, 2007b). If *PpLRL* genes control rhizoid development, it is likely that they may also regulate caulonema development. To determine whether *PpLRL* genes are expressed in the protonema, I stained protonema colonies from the *PpLRL1pro:GUS* and *PpLRL2pro:GUS* lines grown for 2.5 weeks on top of KNOPS media with cellophane discs (**Figure 2-7**). My results indicate that *PpLRL* genes are expressed in the centre of the colony but not at the edge. Since the centre of the colony consists of chloronema filaments while filaments at the edge is more caulonema-like, this results suggests that *PpLRL* genes are more strongly expressed in chloronema filaments than in caulonema filaments. Since caulonema differentiates from chloronema cells, this data suggests that *PpLRL* genes control chloronema development, and may also control the chloronema-to-caulonema transition.

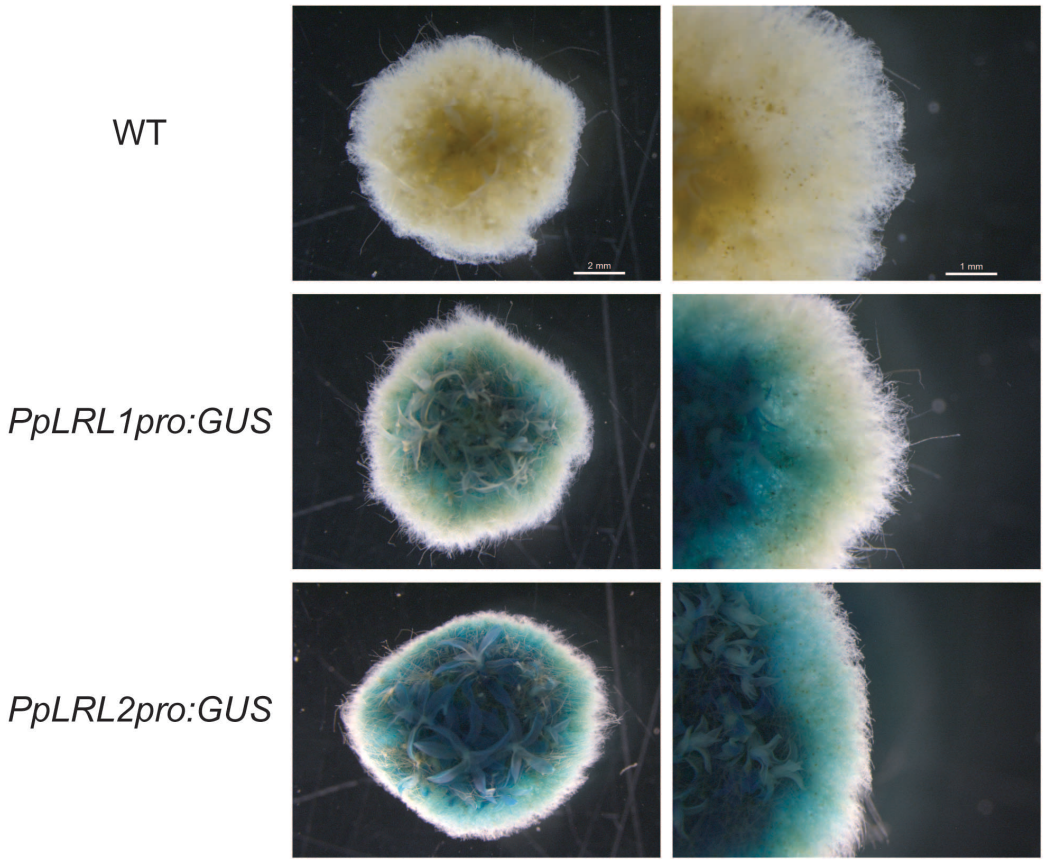


Figure 2-7 Wild type, *PpLRL1pro:GUS* and *PpLRL2pro:GUS* colonies from protonema inocula grown on KNOPS media for 2.5 weeks and stained in GUS-staining solution for 48 hours. In both *PpLRL1pro:GUS* and *PpLRL2pro:GUS* , GUS activity is strongest in the centre of the colony, which is mainly composed of chloronema filaments, indicating that *PpLRL1* and *PpLRL2* are more strongly expressed in chloronema than in caulonema filaments.

2.3.4 *LRL* genes are positive regulators of rhizoid development in *P. patens*

The expression pattern of the *PpLRL* genes is consistent with the hypothesis that *PpLRL* genes control the initiation of rhizoids and caulonema development, but it is also possible that *PpLRL* genes control gametophore and chloronema development. To determine the function of *PpLRL* genes, I generated complete knockout mutants of the moss *P. patens* in which the entire coding sequence was deleted by homologous recombination. Three independent mutant lines for each gene, verified by PCR and Southern blot and shown to contain a single insert at the target locus, were kept for analysis (*Pplrl1-1,2,3* and *Pplrl2-1,2,3*; **Figure 2-1**) . If *LRL* genes positively regulate rhizoid development, I expect to see shorter and/or fewer rhizoids developing on the gametophores of *Pplrl* loss-of-function mutants when compared to WT. To test this, I cultivated wild type and mutant protonema tissues on minimal media with cellophane for 1 month, by which point gametophores are abundant. To ensure that rhizoids on gametophores are at the same developmental stages (gametophore development may not be synchronized on a plate), only gametophores with 12-14 leaves are included in the analysis. Rhizoid length was determined by bundling all the rhizoids in a gametophore and measuring the total length of the bundle (**Figure 2-8**).

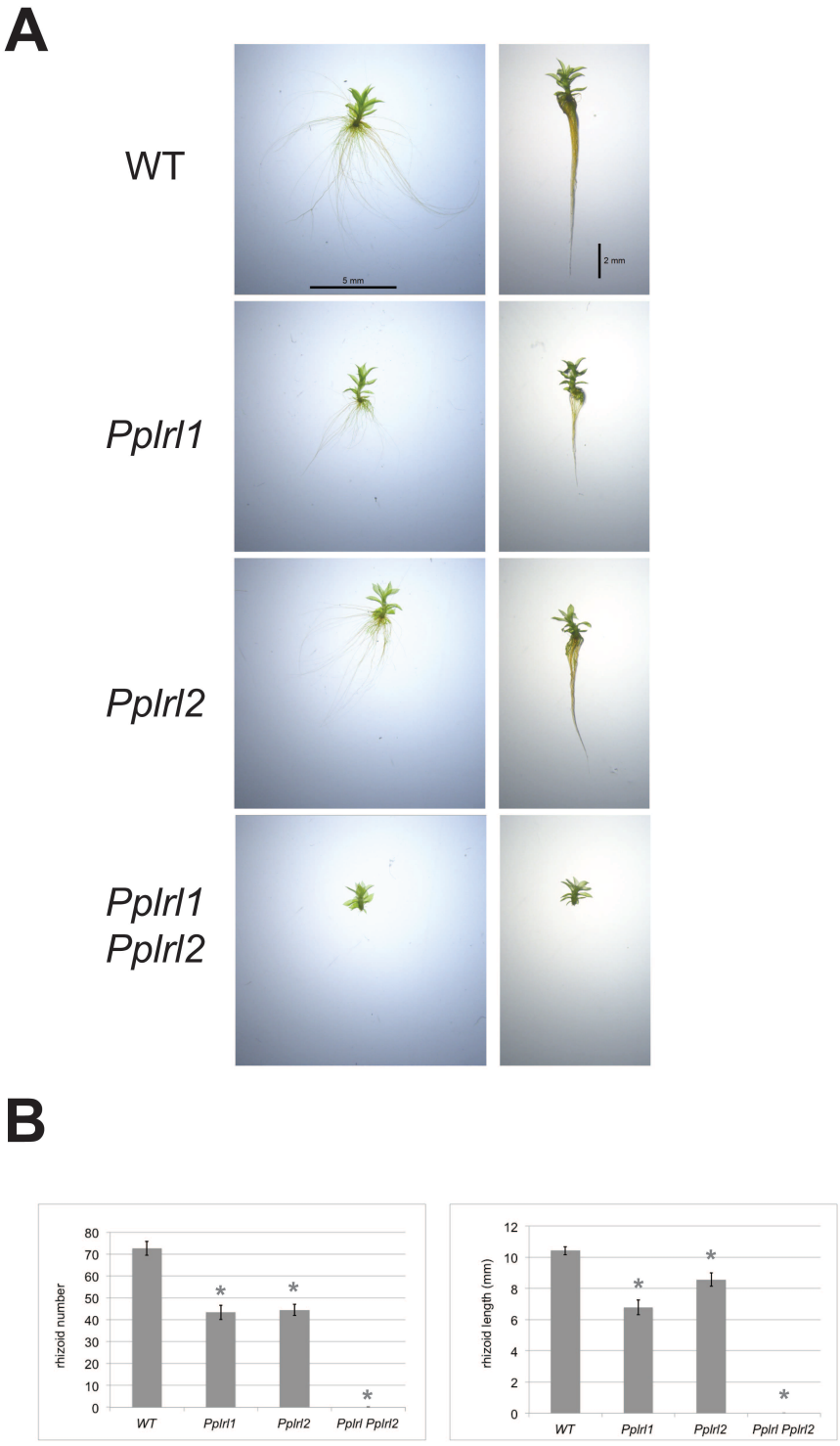
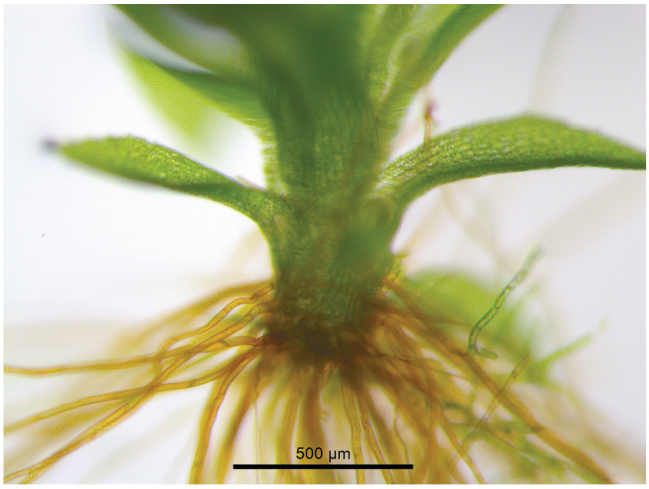


Figure 2-8 Rhizoid phenotypes of *Pplrl* loss-of-function mutants. A) Left panel: gametophores with rhizoid spread out to facilitate counting of rhizoid number; right panel: same gametophores as the left panel but with rhizoids bundled for length measurements. B) Quantification of rhizoid number and rhizoid length. Data were collected from isolated gametophores with 12-14 leaves. Asterisks represent significant difference compared to WT (t-test $P < 0.005$, $n \geq 8$). Error bars represent SE.

In both the *Pplrl1* and *Pplrl2* single mutants, the number of rhizoids is reduced by almost 40% (**Figure 2-8**). The length of rhizoids is also significantly reduced by 35% in the *Pplrl1* mutant and by 18% in the *Pplrl2* mutant. Interestingly, there is a significant difference between rhizoid length in *Pplrl1* and *Pplrl2* (t-test $P<0.05$), suggesting that *PpLRL1* is a more important regulator of rhizoid development than *PpLRL2*. Together these results indicate that *PpLRL1* and *PpLRL2* are positive regulators of rhizoid development in *P. patens*. To determine whether *PpLRL1* and *PpLRL2* have overlapping functions in controlling rhizoid development, I generated *Pplrl1 Pplrl2* double mutants and determined whether a more severe defect can be observed in the rhizoids of the double mutants. The *Pplrl1 Pplrl2* mutants are completely rhizoid-less. Closer inspection confirmed that no rhizoids develop from the epidermis (**Figure 2-9**), and the epidermis appears smooth with no bulges. These results demonstrated that *PpLRL1* and *PpLRL2* are essential for rhizoid development in *P. patens*.

WT



Pplrl1
Pplrl2

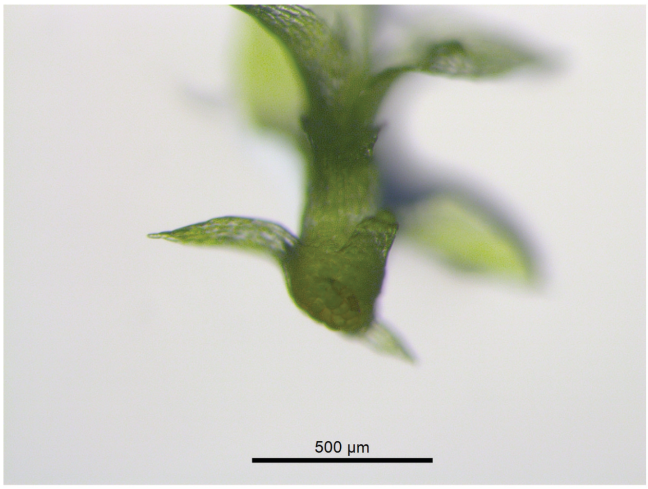


Figure 2-9 *Pplrl1 Pplrl2* double mutants are completely rhizoidless.

2.3.5 *PpLRL1* and *PpLRL2* are positive regulators of caulonema development

Given the role of *PpLRL* genes in specifying the rhizoid fate, I hypothesized that *PpLRL* genes would also positively regulate caulonema development. If true, loss-of-function *Pplrl* mutant colonies should have a reduced diameter of the non-green section at the outer edge of the colony due to defective caulonema development. To test this, I compared the green and non-green diameter of *Pplrl* mutant colonies with wild type (**Figure 2-10**). In the *Pplrl1* and *Pplrl2* single mutants, no significant difference in the green diameter was found compared to WT ($P>0.05$), suggesting that chloronema growth is not significantly different from WT in the single mutants. On the other hand, caulonema growth, as estimated by the non-green diameter of the colony, is significantly reduced in the *Pplrl1* single mutants ($P<0.005$). Although the reduction of caulonema growth is not statistically significant in the *Pplrl2* single mutants ($P>0.05$), the *Pplrl1 Pplrl2 double* mutants are completely caulonema-less. Closer observation of the filaments at the edge of the colonies also shows that caulonema development in *Pplrl1* and *Pplrl2* single are defective and the *Pplrl1 Pplrl2* double mutants completely lack caulonema (**Figure 2-10**). Together these results support the hypothesis that *PpLRL* genes are positive regulators of caulonema development.

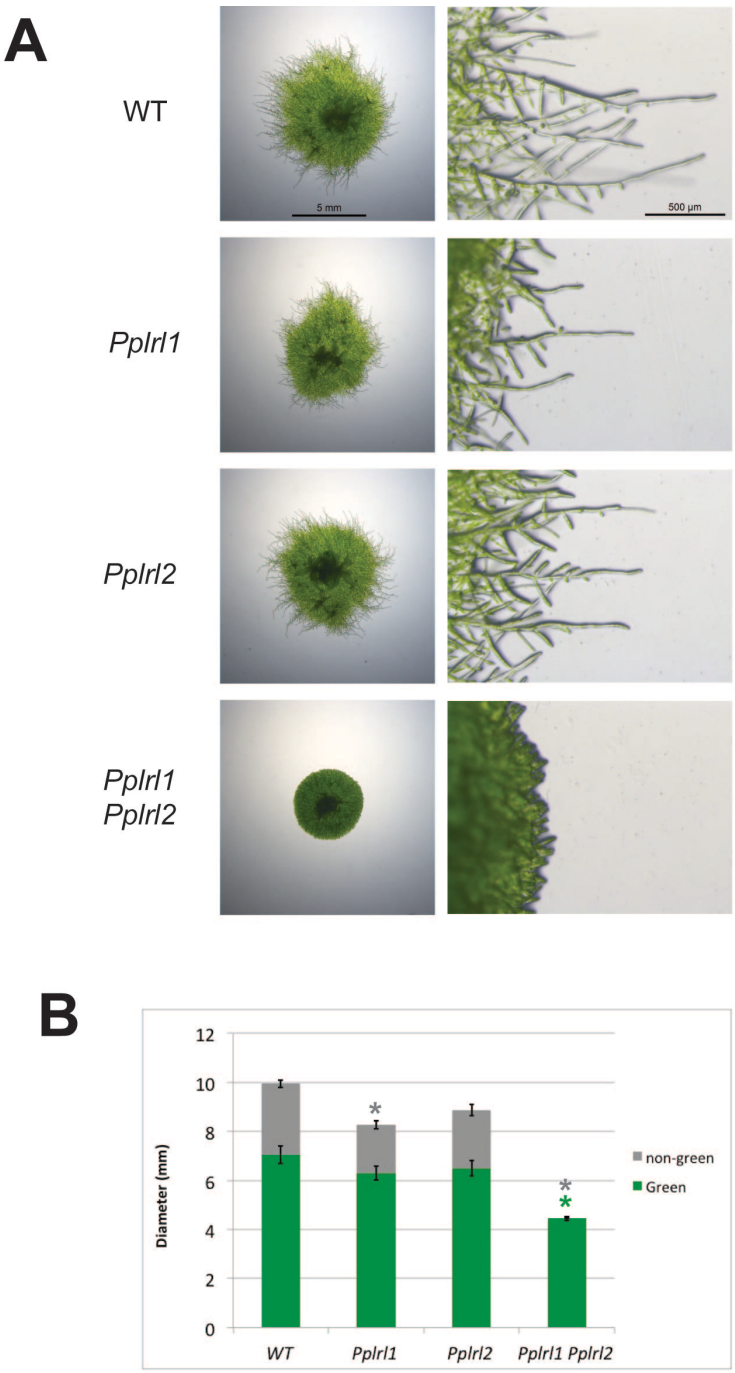


Figure 2-10 *Pplrl* mutants are defective in protonema development. Error bar represents SEM; n=6 to 8. Results of t-tests comparing the mutants with WT are indicated above (for non-green diameter) and below (for green diameter) the error bars. Asterisks indicate that the P-value of t-tests against wild-type is <0.01. Green and Grey asterisks represent comparisons of green and non-green diameter, respectively.

2.3.6 Ancient conserved domains in the *PpLRL* genes are responsible for regulating rhizoid and caulonema development

LRL proteins are characterized by the presence of a highly conserved "LRL" domain at the C-terminus end of the bHLH domain (**Figure 2-11**)(Heim *et al.*, 2003; Pires & Dolan, 2010). If *LRL* genes are conserved regulators of tip-growing rooting cell development, I would expect that these conserved regions would be required for controlling caulonema and rhizoid development in *P. patens*.

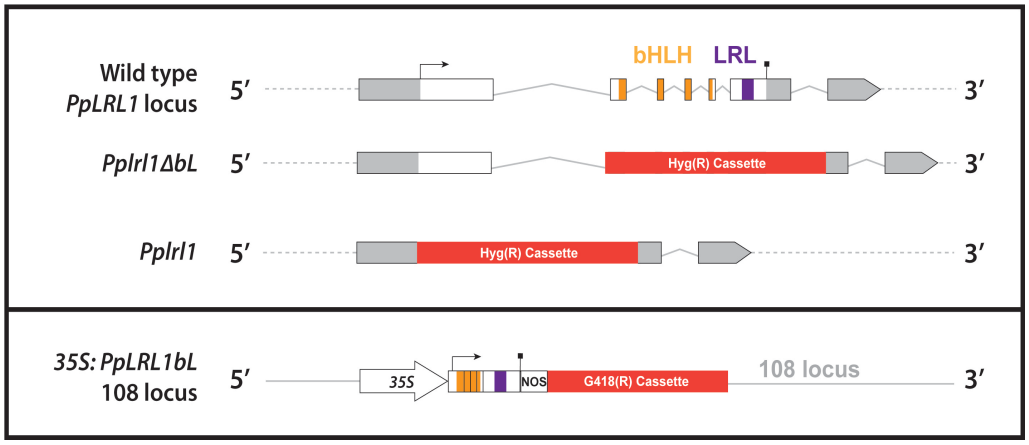


Figure 2-11 Schematic for the *35S:PpLRL1bL* and *Pplrl1ΔbL* lines. The wild type *PpLRL1* gene locus and complete knock out mutants (*Pplrl1*) are also included for comparison. Empty boxes represent exons, curved lines between exons represents introns. Grey boxes represent 5' and 3' untranslated regions (UTRs). Arrows and black squares represents predicted start and stop codon, respectively. 35S: CaMV 35S promoter; NOS: NOS Terminator. Note that the overexpression constructs were knocked into the gene-neutral 108 locus. Similar lines were generated for *PpLRL2*.

To determine if the bHLH and LRL domains are required for *LRL* gene function in controlling rhizoid and caulonema development, I generated partial gene deletion mutants (called *Pplrl1ΔbL* and *Pplrl2ΔbL)* that remove the bHLH and LRL domains while keeping the first exon intact (**Figure 2-11**) . For both *PpLRL1* and *PpLRL2*, the first exon codes for more that half of the mature protein but the bHLH domain is encoded by exon 2,3,4,5 and the LRL domain is encoded by exon 6,

respectively (**Figure 2-11**). Like the *Pplrl* complete gene deletion mutants, The *Pplrl1*Δ*bL* and *Pplrl2*Δ*bL* mutants have defective rhizoid (**Figure 2-12)** and caulonema (**Figure 2-13**) development, suggesting that the bHLH and LRL domain are necessary for *PpLRL* gene function.

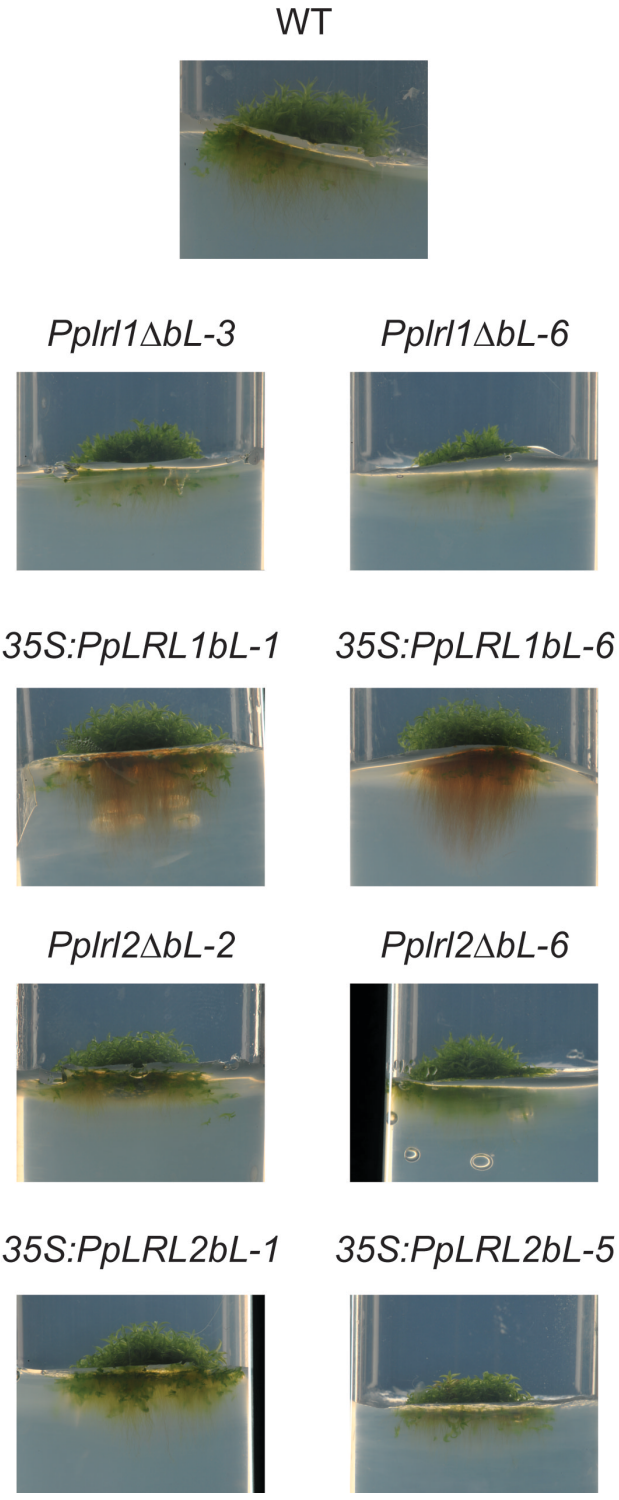


Figure 2-12 Rhizoid development is defective in *PplrlΔbL* and 35S:*PpLRL-bL* lines. Pictures show protonema colonies cultured on KNOPS-GT media in sterile tubes. (Photos courtesy of Mr. Bruno Catarino)

To independently test the hypothesis that *PpLRL* genes are positive regulators of rhizoid and caulonema development I generated over-expression (called *35S:PpLRL1bL* and *35S:PpLRL-bL*) lines expressing a CDS that excludes exon 1 but includes exon 2 to 6, which includes the bHLH and LRL domain (**Figure 2-11**). Consistent with the hypothesis that *PpLRL1* is a positive regulator of rhizoid development, the *35S:PpLRL1bL* plants (*35S:PpLRL1bL-1* and *35S:PpLRL1bL-6*) show enhanced rhizoid development (**Figure 2-12**). Interestingly, *35S:PpLRL2bL* lines show a phenotype that is similar to wild type (*35S:PpLRL2bl-1*). In light of the results described in **Section 2.3.4** (that *Pplrl1* mutant show a more severe rhizoid phenotype than *Pplrl2* mutants), one can interpret this result as indicating that *PpLRL1* is a more important positive regulator of rhizoid development than *PpLRL2*. But this may also be due to the difference in transcript expression level in the different lines. In *35S:PpLRL1bL-1* the expression level of the *PpLRL1bL* transcript is about 16-fold that of wild type, whereas in *35S:PpLRL2bL-1* the *PpLRL2bL* transcript level is only 4-fold (**Figure 2-3**). The fact that *35S:PpLRL2bL-5* show a phenotype that is similar to the *Pplrl2*Δ*bL* loss-of-function mutants may be interpreted by the mechanism of cosupression (Smyth, 1997). The fact that this line also shows inhibited caulonema development (**Figure 2-13**) supports this interpretation.

Caulonema development is also enhanced in the gain-of-function mutants. The non-green diameter of *35S:PpLRL1-bL* and *35S:PpLRL2-bL* colonies is 3 times that of WT but the green diameter is not significantly different from WT (**Figure 2-13**). This suggests that caulonema development is enhanced and that chloronema development is not altered in the gain-of-function mutants.

Taken together, these results support the hypothesis that *PpLRL* genes are positive regulators of rhizoid and caulonema development in *P. patens*. The fact that the conserved bHLH and LRL domains are required for *PpLRL* gene function in regulating rhizoid and caulonema development in *P. patens* suggests that *LRL* genes are ancient regulators of tip-growing rooting cell development and are likely to be positive regulators of rhizoid development in the last common ancestors of mosses and angiosperms.

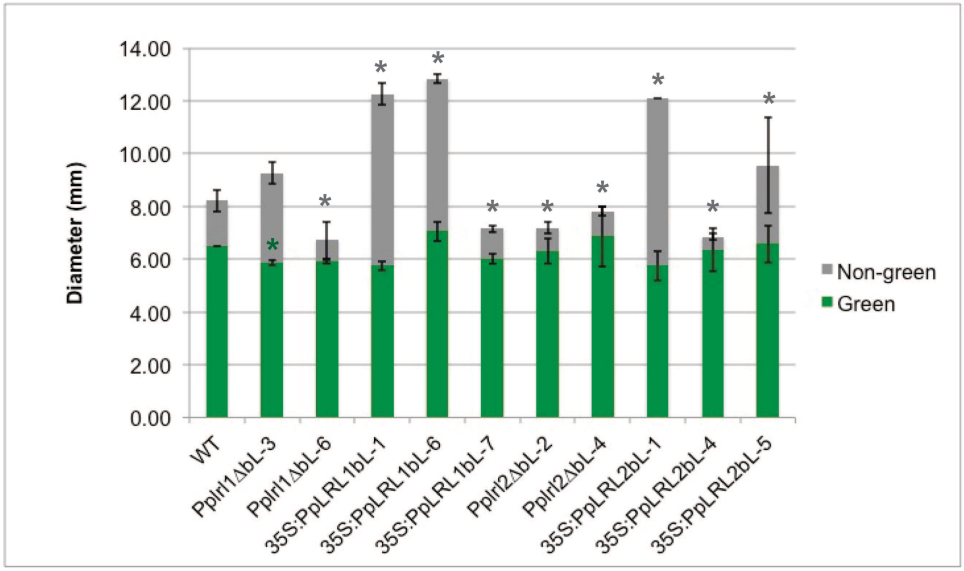


Figure 2-13 Diameter of green and non-green area of colonies (27d old. Error bars represents S.D. n=3 except in *35S:PpLRL2bL-4*, where n=2. Asterisks indicate that the P-value of t-tests against wild-type is <0.01. Green and Grey asterisks represent comparisons of green and non-green diameter, respectively.

2.3.7 *PpLRL* genes are not general regulators of tip growth

My results show that *PpLRL* genes are positive regulators of rhizoid and caulonema differentiation. However, it is formally possible that *LRL* genes are regulators of tip

growth in general. If true, I would expect to observe defects in both chloronema and caulonema development, since both chloronema and caulonema grow by tip growth (Menand *et al.*, 2007a). To test this, I used time-lapse microscopy to measure the growth rate of young secondary chloronema filaments, where differentiation into caulonema filaments is minimal (**Figure 2-14**). The characteristic growth rate of chloronema, as reported in the literature, is about 6 µm per hour (in contrast to caulonema, which grows at about 20 µm hour) (Menand *et al.*, 2007a). My results detected a slightly higher rate for chloronema growth (8 µm per hour) in wild type plants. However, I found no significant difference (P>0.05) in chloronema elongation rate between wild type and the *Pplrl1*, *Pplrl2,* or the *Pplrl1 Pplrl2* mutants (**Figure 2-14**). This result suggests that *PpLRL* genes do not regulate chloronema growth rate. Because chloronema elongate by tip growth, this result shows that general tip-growing mechanisms are unlikely to be defective in the *Pplrl* mutants. These data suggest that *PpLRL* genes are specific regulators of rhizoid and caulonema development in *P. patens*.

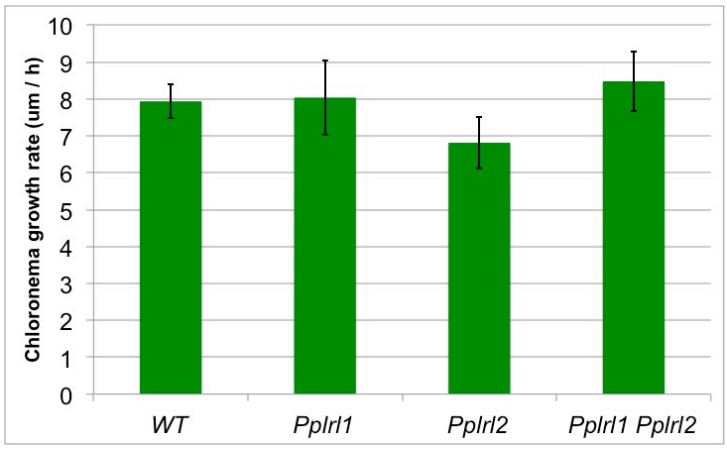


Figure 2-14 Chloronema elongation rate is not affected in the *Pplrl* loss-of-function mutants. Error bar represents SEM; n=3

2.4 Discussion

In this chapter, I showed that the *LRL* genes comprise a group of conserved bHLH transcription factors in most land plants and that the *LRL* genes in the moss *P. patens* are positive regulators of rhizoid and caulonema development. *PpLRL* genes, however, are not strongly expressed in rhizoids or caulonema. Instead, they are expressed in the base of the young gametophores, and in chloronema cells. This suggests that *PpLRL* genes are likely to act early in rhizoid and caulonema development, possibly by regulating their initiation in the epidermal cells at the base of the gametophore and in chloronema cells. Given that *PpLRL* genes are positive regulators of rhizoid and caulonema development, changes in the regulation or expression of *LRL* genes in the ancestor of land may have played important roles in the evolution of rooting systems in land plants.

LRL proteins have diverged substantially in land plants, radiating into three distinct classes in the angiosperms, but they are characterized by the presence of the highly conserved bHLH and LRL domain. In *P. patens*, over-expression of truncated transcripts encoding "minimal" versions of the PpLRL protein that include the bHLH and LRL domains was sufficient to induce increased caulonema development. These results suggest that the ancient function of LRL proteins likely include the regulating of tip-growing rooting cells.

Because it has been shown that *LRL* genes control root hair development in angiosperms (Ding *et al.*, 2009; Karas *et al.*, 2009; Bruex *et al.*, 2012), these results support the hypothesis that one of the main functions of ancestral *LRL* genes in the last common ancestor of mosses and angiosperms is to control tip-growing rooting cell development. Recruitment of *LRL* genes from the gametophore to the

sporophyte generation, therefore, may have contributed to the evolution of root hairs and the increasing morphological complexity in the sporophyte generation in vascular plants.

3 *PpLRL* genes mediate auxin and low phosphate response in tip-growing rooting cells in *P. patens*

3.1 Introduction

Auxin and phosphate availability have profound effects on tip-growing rooting cell development in land plants. In *A.* thaliana, application of exogenous auxin increases the density and length of root hairs, while shortage of auxin supply or mutations in the auxin signalling pathway inhibits root hair development (Knox *et al.*, 2003; Lee & Cho, 2006; Jones *et al.*, 2009; Yi *et al.*, 2010; Pires *et al.*, 2013). Root hair development is also induced by low phosphate availability in *A. thaliana,* rice, spinach, tomato, and rape. (Foehse & Jungk, 1983; Ma *et al.*, 2001; Bates & Lynch, 2006; Shen *et al.*, 2012). Similar responses to auxin and low phosphate can be observed in *P. patens*. Application of exogenous auxin promotes rhizoid and caulonema development, while mutants in the auxin biosynthesis or auxin signalling pathways show defective caulonema development (Ashton *et al.*, 1979; Ashton, 1998; Eklund *et al.*, 2010b; Prigge *et al.*, 2010; Jang & Dolan, 2011; Jang *et al.*, 2011; Lavy *et al.*, 2012). Similar to the angiosperms, low phosphate also stimulates rhizoid and caulonema development in *P. patens* (Wang *et al.*, 2008). Together, these data suggest that auxin and low phosphate are common positive regulators of tip-growing rooting cell development in land plants.

Interaction between auxin and low phosphate signalling has been demonstrated in *A. thaliana* and rice (López-Bucio *et al.*, 2002; Jain *et al.*, 2007; Pérez-Torres *et al.*, 2008; Miura *et al.*, 2011; Shen *et al.*, 2012). In *A. thaliana*, one pathway of auxin signalling involves auxin binding to the SCF^{TIR1} complex, where TIR1 is an auxin receptor (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005). This complex targets

Aux/IAA proteins for ubiquitin-mediated degradation. Degradation of Aux/IAA proteins results in de-repression of auxin response factors (ARFs). De-repressed ARFs can either activate (ARF^A) or repress (ARF^R) the expression of auxin-responsive genes (Teale *et al.*, 2006; Del Bianco & Kepinski, 2011). In *A. thaliana*, low phosphate induces the expression of the auxin receptor *TIR1* and leads to increased degradation of the Aux/IAA protein AXR3/IAA17 (Pérez-Torres *et al.*, 2008). This suggested that auxin sensitivity is increased under low phosphate (Pérez-Torres *et al.*, 2008). The auxin response factor *AtARF19* is also required for low phosphate-induced lateral root formation in *A. thaliana* root (Pérez-Torres *et al.*, 2008). *AtSIZ1*, which regulates auxin distribution in the root, also regulates low phosphate response (Miura *et al.*, 2011). In rice, *OsARF16* gene function is required for both auxin- and low phosphate-induced root hair elongation (Shen *et al.*, 2012), These data suggest that auxin and low-phosphate signalling pathways interact to control root hair development in angiosperms. In *P. patens*, major components of the TIR1 auxin signalling pathway, including 4 TIR1/AFB auxin receptors, 2 (or 3) Aux/IAA proteins, and 14 ARFs are present in the genome (Rensing *et al.*, 2008; Prigge *et al.*, 2010), suggesting that similar mechanisms may operate in *P. patens*. Indeed, knocking down the TIR1 homologs *PpAFB1* to *PpAFB4* in *P. patens* resulted in suppression of caulonema development (Prigge *et al.*, 2010). Exogenous auxin induce the expression of *PpIAA* genes and mutants that show defect degron motif in *PpIAA* genes are insensitive to auxin and show phenotypes that are comparable to those of the *Ppafb* knockdown mutants (Prigge *et al.*, 2010). These results showed that auxin signalling in the chloronema, which are necessary for the development of caulonema, are mediated through the *PpIAA* and *PpAFB* proteins.

In chapter 2, I showed that *PpLRL* genes are positive regulators of caulonema and rhizoid development in *P. patens*. In this chapter, I tested the hypothesis that *PpLRL* genes mediate auxin and low phosphate response in tip-growing rooting cells in *P .patens*.

3.2 Materials and methods

3.2.1 Plant material

I am grateful to Dr Katarina Lundberg from Prof. Eva Sundberg's group (Swedish University of Agricultural Sciences, Sweden) for providing the *Ppshi1*, *Ppshi2* and the *PpSHI1* overexpression lines (Eklund *et al.*, 2010b). Dr Michael Prigge from Prof Mark Estelle's group (University of California San Diego, USA) provided the *Ppiaa1A-113, Ppiaa1B-112* and *Ppiaa2-183* lines (Prigge *et al.*, 2010).

3.2.2 Auxin and low phosphate treatment

For auxin treatments, plants were grown on cellophane discs on KNOPS-GT media (as described in Chapter 2) supplemented with 0 uM, 0.1 uM or 1 uM of 1-naphthaleneacetic acid (NAA) in dimethyl sulfoxide (DMSO).

Both +P and –P media is based on the KNOPS-GT media described in Chapter 2. The high phosphate media (+P) is equivalent to the KNOPS-GT ('minimal') media supplemented with 1mM MES. The low phosphate media (-P) is prepared by replacing the KH_2PO_4 buffer in the high phosphate (+P) media with 0.918mM of K_2SO_4.

3.2.3 qRT-PCR

To determine steady-state *PpLRL* transcript level in auxin-treated plants, macerated protonema tissues were grown on cellophanes discs on minimal media with or without auxin (1µm NAA) for 1 week. To determine steady-state *PpLRL* transcript level in low phosphate, 10-day-old macerated protonema tissues grown on

cellophane discs on –P media were used. Macerated protonema tissues grown on minimal media were also used to determine the steady-state *PpLRL* transcript levels in *Ppshi1,* Ppshi2, *OXSHI1-5*, *Ppiaa1A-113, Ppiaa1B-112* and *Ppiaa2-183* mutants. RNA extraction, cDNA synthesis and qRT-PCR are carried out as described in Chapter 2. Primers used are given in the **Appendix A5**.

3.3 Results

3.3.1 Auxin positively regulates the expression of *PpLRL1* and *PpLRL2*

Auxin is a positive regulator of rhizoid and caulonema development in *P. patens* (Ashton, 1998; Sakakibara *et al.*, 2003; Eklund *et al.*, 2010b; Prigge *et al.*, 2010; Jang & Dolan, 2011; Jang *et al.*, 2011; Lavy *et al.*, 2012; Pires *et al.*, 2013). Because I showed that *PpLRL* genes positively regulate rhizoid and caulonema development (Chapter 2), I hypothesized that auxin may induce rhizoid and caulonema development by positively regulating *PpLRL1* and *PpLRL2* expressions. To test this, I used qRT-PCR to compare the steady-state transcript levels of *PpLRL1* and *PpLRL2* in 1-week-old protonema tissues grown on minimal media with or without 1 μM NAA. My results indicate a small increase in *PpLRL* level in plants treated with auxin **(Figure 3-1)**, but the difference between treated plants and untreated plants is less than 2-fold, a minimum threshold for establishing a significant differential expression using qRT-PCR. This suggests that, in these conditions, the *PpLRL* transcript levels are not significantly increased by treatment with exogenous auxin.

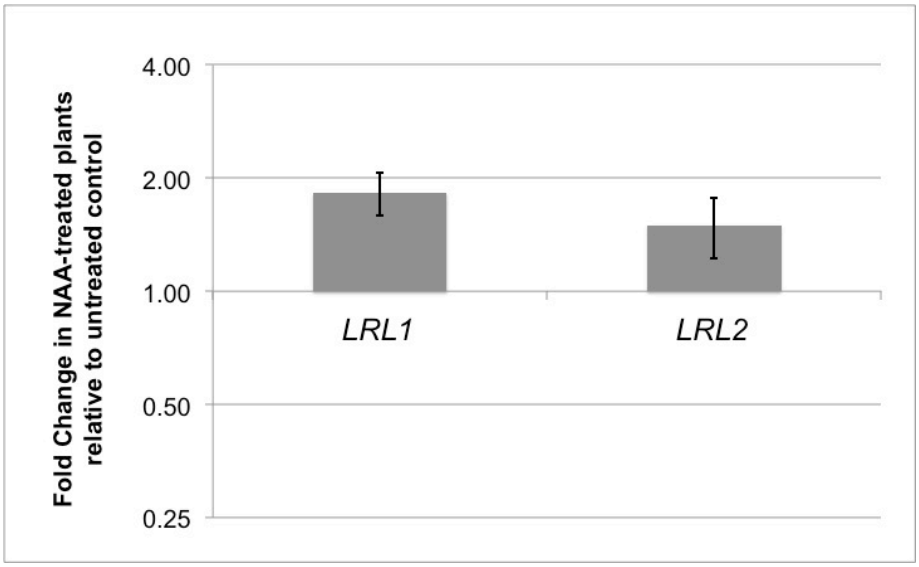


Figure 3-1 Transcript level of *PpLRL1* and *PpLRL2* in NAA-treated plants relative to untreated control. Error bars represent S.D.

As a second test for the hypothesis that auxin positively regulates the expression of *PpLRL1* and *PpLRL2*, I determined the expression of *LRL* genes in the protonema of mutants defective in endogenous auxin biosynthesis, including the *Ppshi1, Ppshi2*, and *OXSHI1-5* mutants (Eklund *et al.*, 2010b). Direct measurement of auxin level in gametophores or the gametophore apex in these mutants, using gas chromatography-mass spectrometry, showed that *PpSHI1* and *PpSHI2* are positive regulators of auxin biosynthesis (Eklund *et al.*, 2010b). Therefore, I expect that endogenous auxin level would be lower in the *Ppshi1* and *Ppshi2* mutants compared to wild type, but higher in the *OXSHI1-5* mutants that overexpress the *PpSHI1* gene. Thus, if auxin positively regulates *PpLRL* gene expression, I would expect to observe that the steady-state transcript levels of *PpLRL1* and *PpLRL2* would be decreased in the *Ppshi1* and *Ppshi2* mutants, but increased in the *OXSHI1-5* mutant.

My qRT-PCR results show that the steady-state transcript levels of *PpLRL1* and *PpLRL2* are decreased by 4-folds in the *Ppshi2* mutants (**Figure 3-2**). However, no significant differences in the steady-state transcript levels of *PpLRL* genes were detected in either the *Ppshi1* or the *OXSHI1-5* mutants relative to wild type (**Figure 3-2**). These results indicate that *PpSHI2* positively regulates *PpLRL* genes while *PpSHI1* does not regulate the expression of *PpLRL* genes. One interpretation of these results may be that *PpSHI1* and *PpSHI2* contributed differently to the synthesis of endogenous auxin in the protonema (with *PpSHI2* playing a more prominent role), and therefore the *Ppshi2* mutants have lower endogenous auxin levels in the protonema than *Ppshi1* mutants. An alternative explanation is that *PpSHI2* (but not *PpSHI1*), being a transcription factor, directly regulates the expression of *PpLRL* genes. Comparing the endogenous auxin level in the protonema of *Ppshi1, Ppshi2*, and *OXSHI1-5* mutants should help distinguish between these possibilities.

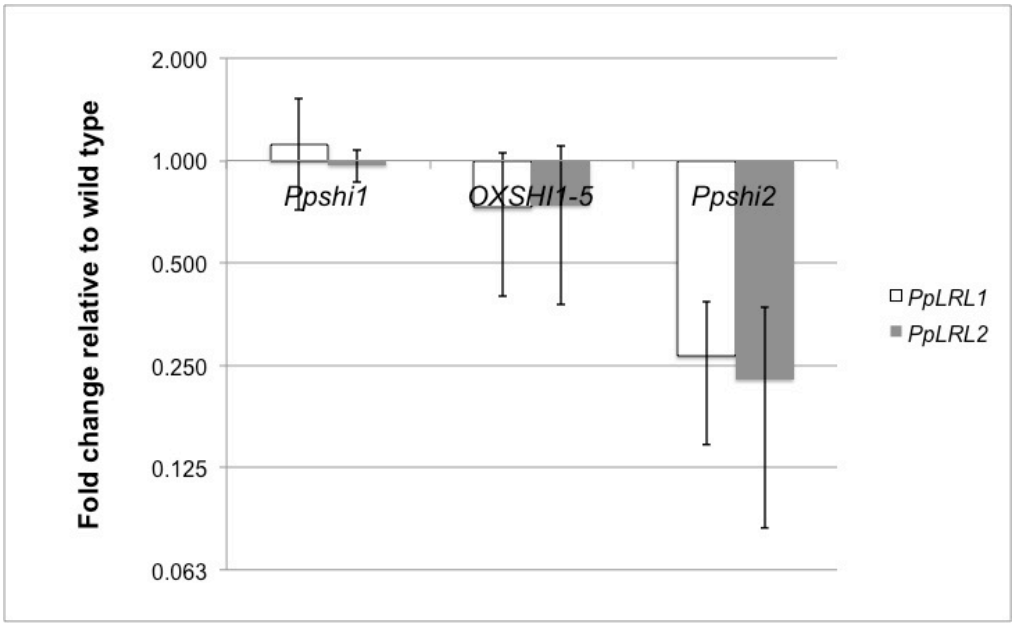


Figure 3-2 Steady state level of *PpLRL1* and *PpLRL2* are lower in the *Ppshi2* mutant but is not affected in the *Ppshi1* or *OXSHI1-5* mutants. Error bars represent S.D.

Reasoning that qRT-PCR analyses may not detect small changes in expression level, or changes in the expression pattern of auxin-responsive genes, I utilized the *PpLRL1pro:GUS* and *PpLRL2pro:GUS* reporter lines and test whether auxin treatment would induce the activity of the *PpLRL1* and *PpLRL2* promoters. *PpLRL1pro:GUS* and *PpLRL2pro:GUS* plants were grown in minimal media supplemented with 0.1µm NAA for 1 month and checked for GUS signal after 24h of staining. Under these conditions, plants without auxin-treatment do not express GUS, suggesting that under this condition, endogenous auxin does not significantly increase the expression of the *PpLRL* genes. However, in plants treated with auxin, GUS signal is detected in chloronema and in the developing buds of both *PpLRL1pro:GUS* and *PpLRL2pro:GUS* (**Figure 3-3**). This suggests that elevated exogenous auxin level induces *PpLRL* gene expression in the chloronema and in the developing buds. This expression pattern is consistent with that observed in *PpLRL1pro:GUS* and *PpLRL2pro:GUS* plants grown under high-energy condition (Chapter 2). Together these results suggest that auxin positively regulates *PpLRL1* and *PpLRL2* expressions in chloronema and the developing buds. The fact that auxin does not induce *PpLRL* expression in all tissues suggests that *PpLRL* expression may be regulated by other factors. Together, these results demonstrated that the expression of *PpLRL* genes are positively regulated by auxin but is likely to be regulated by additional factors.

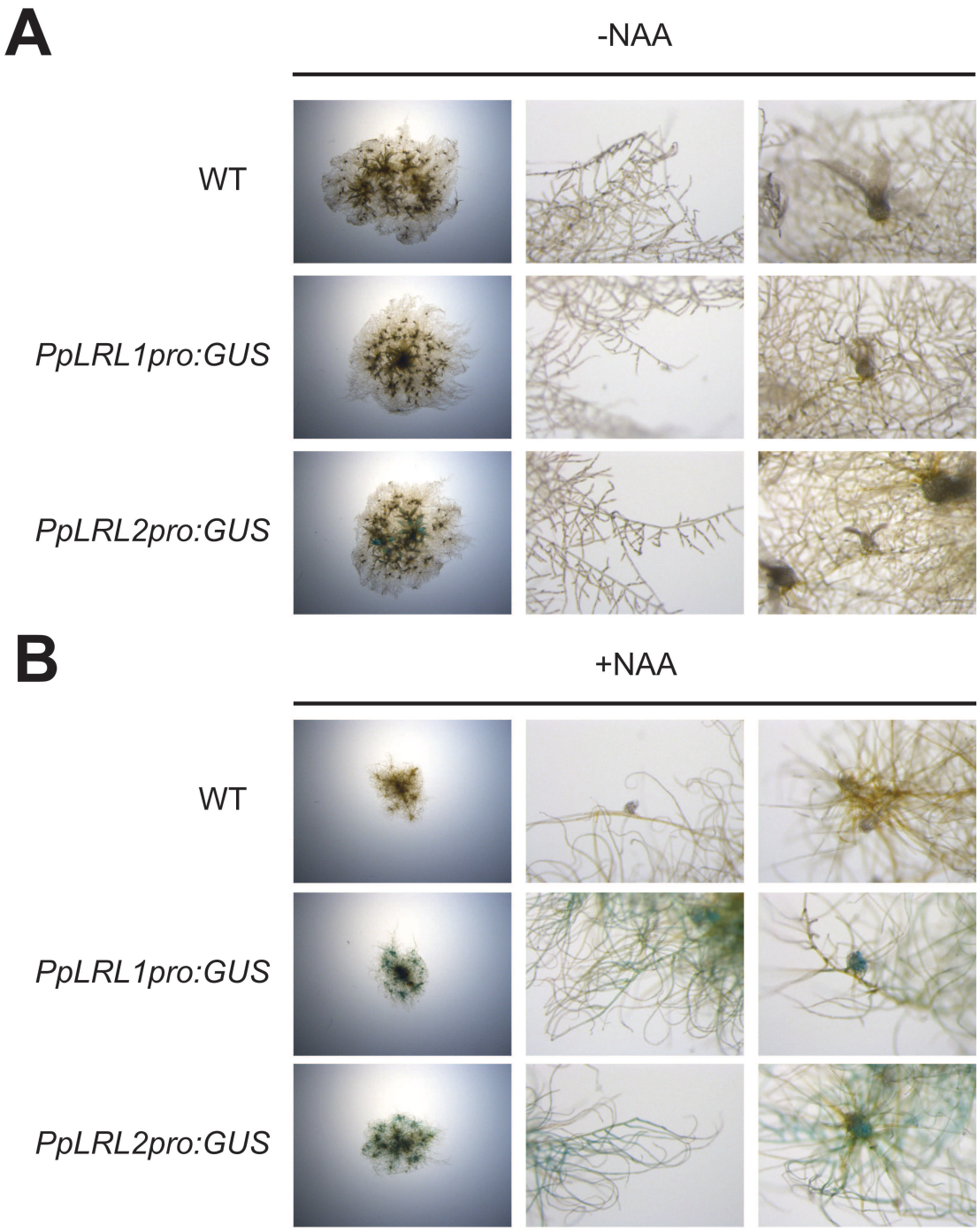


Figure 3-3 GUS staining pattern of *PpLRL1* and *PpLRL2* in protonemata colonies cultivated on minimal media with (A) no auxin (-NAA) or with (B) 0.1uM NAA (+NAA) for 1 month. Colonies were stained for 24 hours; at this point GUS signal cannot be detected in the -NAA plants is clearly visible in chloronema cells and the developing buds in plants grown in the presence of auxin (+NAA).

3.3.2 *PpIAA1A* is involved in the regulation of *PpLRL* gene expression

In *P. patens*, PpIAA proteins are required for caulonema development (Prigge *et al.*, 2010). In the *Ppiaa1A*, *Ppiaa1B* and *Ppiaa2* gain-of-function mutants, which produce mutant forms of Ppiaa proteins that cannot be effectively targeted for degradation (thus constitutively repressing auxin signalling), caulonema development is highly defective (Prigge *et al.*, 2010). Since *PpLRL* gene activity are required for caulonema development, I hypothesized that *PpLRL* genes may be one of the downstream targets of PpIAA proteins.

To test this, I determined the steady-state transcript levels of *PpLRL1* and *PpLRL2* in the *Ppiaa1A, Ppiaa1B,* and *Ppiaa2* mutants using qRT-PCR. My results showed that the steady-state transcript levels of *PpLRL1* and *PpLRL2* are increased in the *Ppiaa1A* mutant compared to wild type, but are not affected in the *Ppiaa1B* or the *Ppiaa2* mutants (**Figure 3-4**). This indicates that PpIAA1A, but not PpIAA1B or PpIAA2, regulates the expression of *PpLRL* genes. However, the fact that the steady-state levels of PpLRL genes are increased in the *Ppiaa1A* mutant highlights the complex nature of auxin response. The *Ppiaa1A* mutant produces a mutant form of Ppiaa1A protein that cannot be degraded in the presence of auxin; in other words the *Ppiaa1A* mutant is auxin insensitive as the auxin response pathway including the ARFs downstream of Ppiaa1A is constitutively repressed. Since this constitutive repression led increased steady-state transcript levels of *PpLRL* genes, the model of Aux/IAA-mediated auxin signalling would suggest that the mutant form of Ppiaa1A activates *PpLRL* gene expression. This means that when PpIAA1A is degraded (in above-threshold auxin level), the transcription level of *PpLRL* should decrease. This is inconsistent with the observations that auxin positively regulates *PpLRL* expression and the fact that both auxin and *PpLRL* genes are positive regulators of

caulonema development (see above and Chapter 2). However, these results may be explained by the complex nature of auxin signalling in *P. patens.* Elucidating the localization and the binding specificity of PpIAA proteins and PpARF proteins should shed light on this issue. Together, these results suggest that auxin positively regulates the expression of *PpLRL* genes, even though the signalling pathways may be complex.

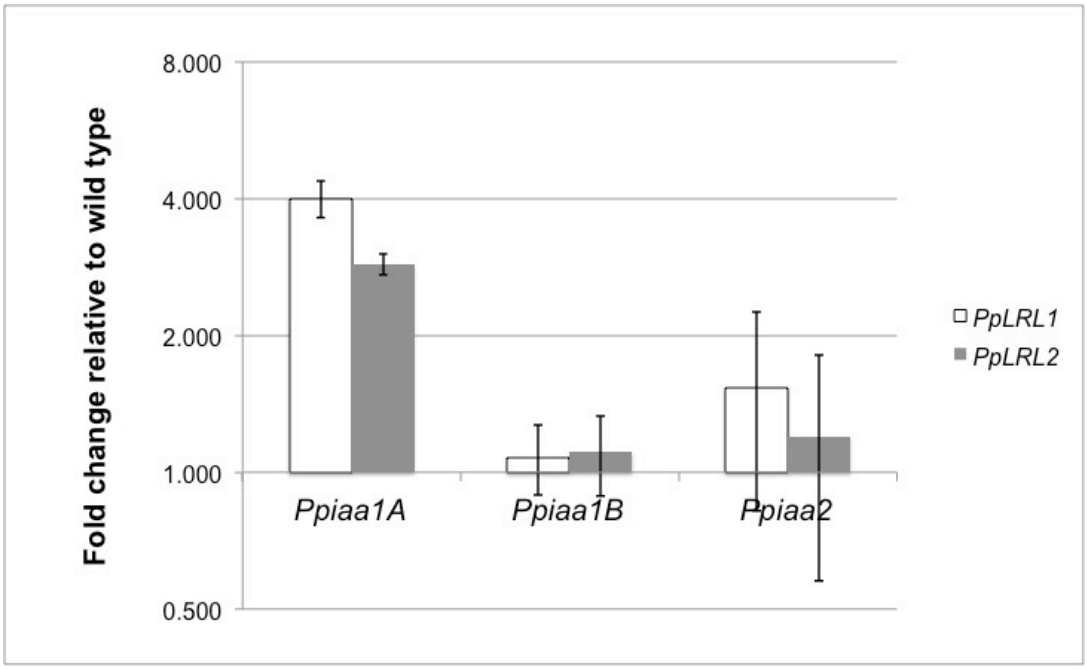


Figure 3-4 Steady state level of *PpLRL1* and *PpLRL2* are higher in the *Ppiaa1A* mutant. Error bars represent SD.

3.3.3 Auxin response in the protonema requires *PpLRL* gene function

If *PpLRL* genes mediate auxin response in caulonema and rhizoids in *P. patens* , I would expect to observe defective auxin response in the *Pplrl* loss-of-function mutants. To test this, I grew loss-of-function *Pplrl* mutants in minimal media with or without exogenous auxin (0.1μM NAA) and observed the colonies after 2.5 weeks.

In wild type plants, auxin treatment strongly inhibits protonema growth (colony diameter significantly decreased by 52%) (**Figure 3-5**) but promotes the chloronema-to-caulonema transition (Ashton *et al.*, 1979; Ashton, 1998; Jang & Dolan, 2011). In the *Pplrl1* single mutants, protonema growth was strongly inhibited by auxin (colony diameter significantly decreased by 35%) (**Figure 3-5**). No significant difference was found in the colony diameter between wild type and *Pplrl1* single mutant under auxin treatment, suggesting that auxin response is not defective in the *Pplrl1* mutants. However, in auxin-treated *Pplrl2* single mutant, colony diameter was significantly larger than wild type ($P<0.01$) (**Figure 3-5**). This suggests that auxin response is defective in the *Pplrl2* mutants. The fact that the defect in auxin response is more severe in *Pplrl2* mutants suggests that *PpLRL2* may be a more important player in auxin response, at least in protonema growth, than *PpLRL1*. In the *Pplrl1 Pplrl2* double mutants, there was no significant difference between treated and untreated plants in colony diameter ($P > 0.05$) **(Figure 3-5**), and no caulonema were produced in the auxin-treated plants (**Figure 3-6**), suggesting that auxin treatment cannot rescue the defective caulonema phenotype in the *Pplrl1 Pplrl2* mutants. Together these results suggest that *PpLRL* gene activity is required for auxin response in the protonema of *P. patens*.

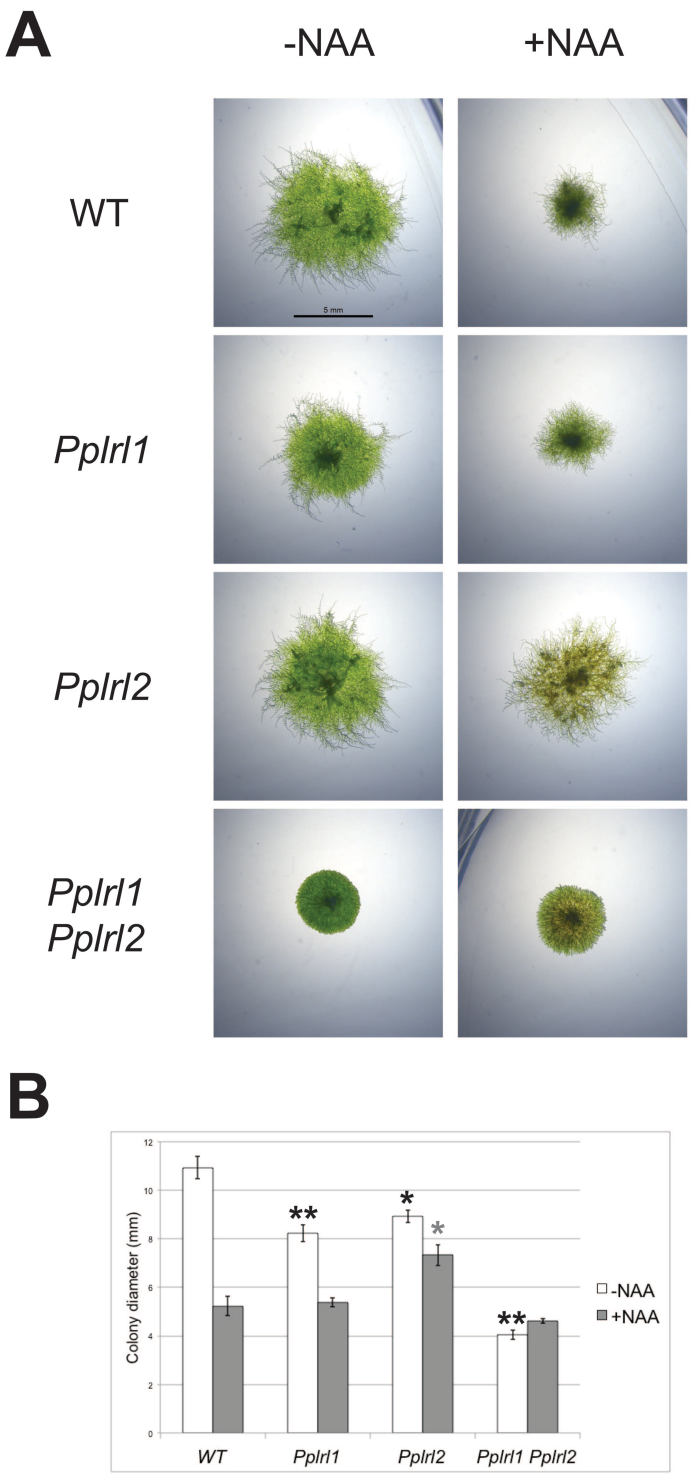


Figure 3-5 *Pplrl* mutants are resistant to exogenous auxin. A) Phenotypes of 2.5-week old protonema colony with or without 0.1 µM NAA. B) Quantification of colony diameter. Error bars represent SE. Asterisks represents significant results (*P<0.05, **P<0.01) of t-test between WT and the *Pplrl* mutants with auxin treatment (Grey asterisks) or without auxin treatment (black asterisks) ; n=4 except in *Pplrl1 Pplrl2* -NAA, where n=3.

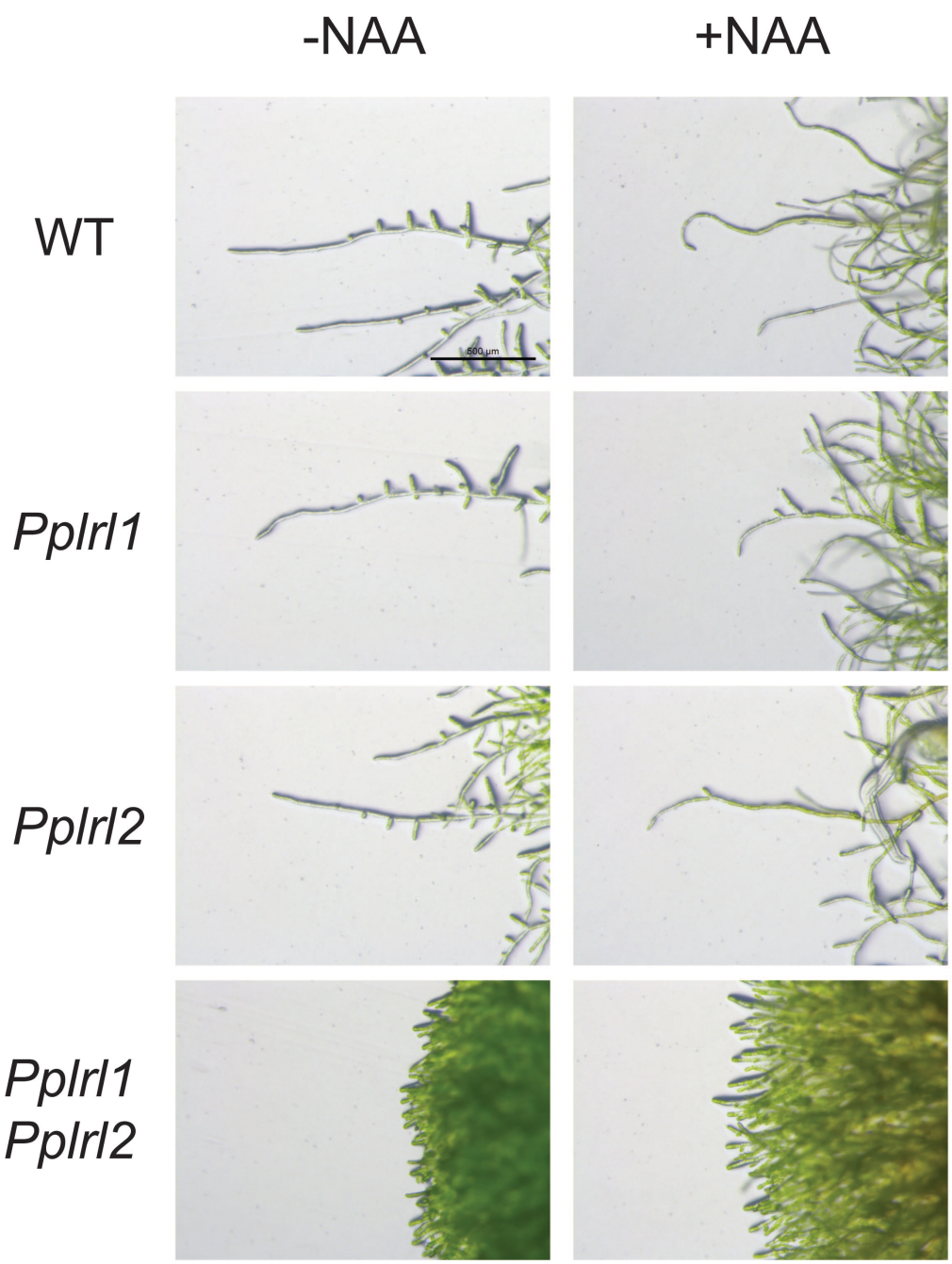


Figure 3-6 No caulonema develop in the *Pplrl1 Pplrl2* double mutants.

3.3.4 The Induction of rhizoid development by auxin requires *PpLRL* gene function

Auxin is a positive regulator of rhizoid development in *P. patens* (Ashton, 1998; Sakakibara *et al.*, 2003; Jang *et al.*, 2011). In wild type plants, auxin induces the production of ectopic rhizoid development in the gametophore, leading to the production of a leafless gametophore with numerous rhizoids developing laterally from along the height of the gametophore (**Figure 3-7**). In auxin-treated *Pplrl1* and *Pplrl2* single mutants, leafless gametophores with ectopic rhizoids can be observed, but the rhizoids appear to be shorter than wild type plants under the same condition (with auxin treatment) (**Figure 3-7**). However, in the *Pplrl1 Pplrl2* double mutants, no rhizoids can be observed on the leafless gametophores (**Figure 3-7**). These results suggest that *PpLRL* gene function is required for auxin to induce rhizoid development.

Together, these results showed that *PpLRL* genes are required for auxin response in protonema and rhizoid development, and thus support the hypothesis that auxin response in *P. patens* in protonema and rhizoid development, are mediated via *PpLRL* genes.

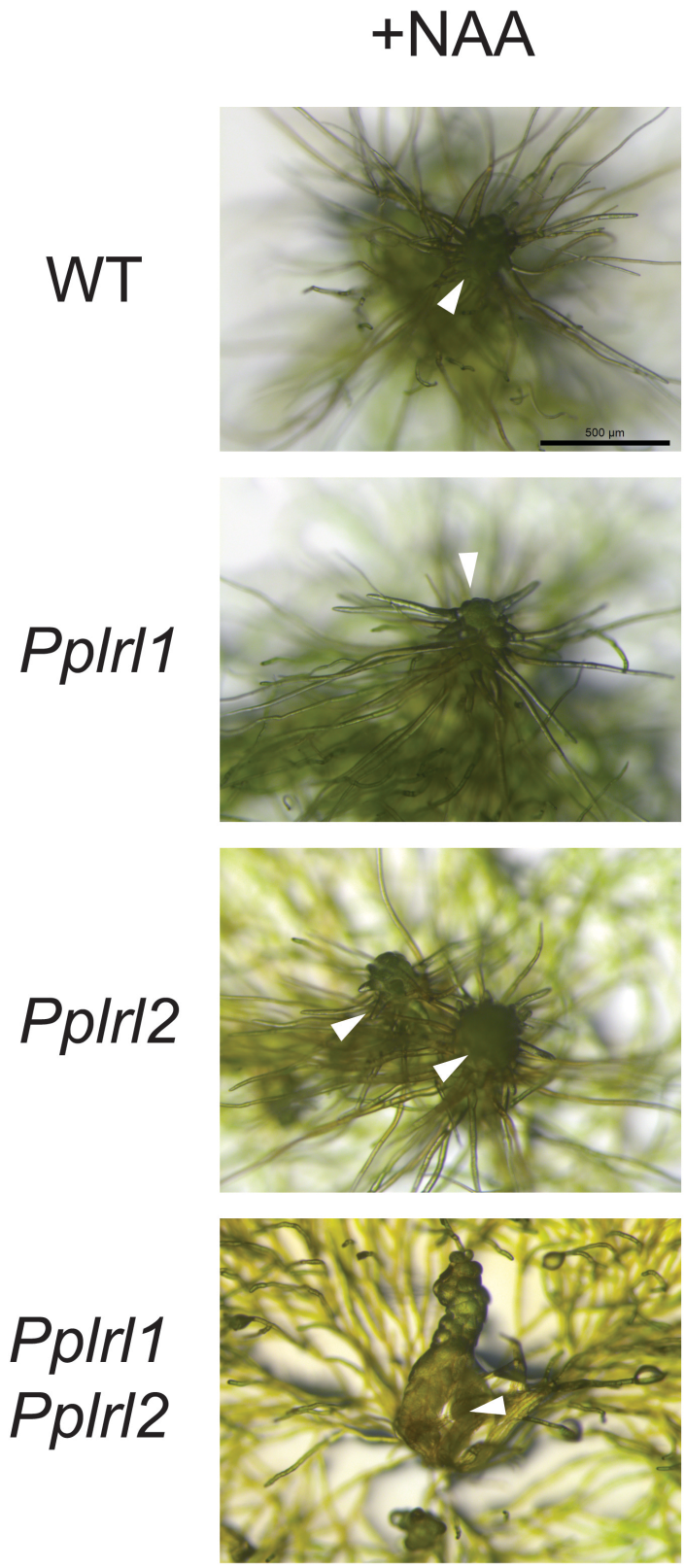


Figure 3-7 Auxin treatment induces ectopic rhizoid development in wild type and *Pplrl1* and *Pplrl2* single mutants but not in *Pplrl1 Pplrl2* double mutants. White arrowheads indicate the position of the gametophores.

3.3.5 Low phosphate response in the protonema requires PpLRL gene function

Low phosphate induces caulonema development in wild type *P. patens* (Wang *et al.*, 2008). Given that *PpLRL* genes positively regulate caulonema development I hypothesized that *PpLRL* gene activity is required for low phosphate response in caulonema development. To test this, I grew *Pplrl* loss-of-function mutants on high (+P) and low (-P) phosphate media and observe the development of the protonema colony after 2.5 weeks (**Figure 3-8**). Taking into account that mutations in *PpLRL* genes affect caulonema development itself (Chapter 2), I used the increase in caulonema: chloronema ratio in each colony (estimated by the green and non-green diameter) as a proxy to determine whether phosphate response is defective in the protonema. Low phosphate treatment led to a 52% increase in the caulonema : chloronema ratio in both wild type (from 0.260 to 0.394) and *Pplrl2* single mutant (from 0.281 to 0.428) colonies, suggesting that low phosphate response is not defective in the *Pplrl2* single mutants. However, in the *Pplrl1* single mutant, low phosphate treatment led to a 97% increase in the ratio (from 0.243 to 0.479), suggesting that low phosphate response is more highly induced in the *Pplrl1* single mutants than wild type. In the *Pplrl1 Pplrl2* double mutants, low phosphate treatment cannot rescue the no-caulonema phenotype. Together, these results suggest that *PpLRL* gene function is required for normal low phosphate response in *P. patens* protonema.

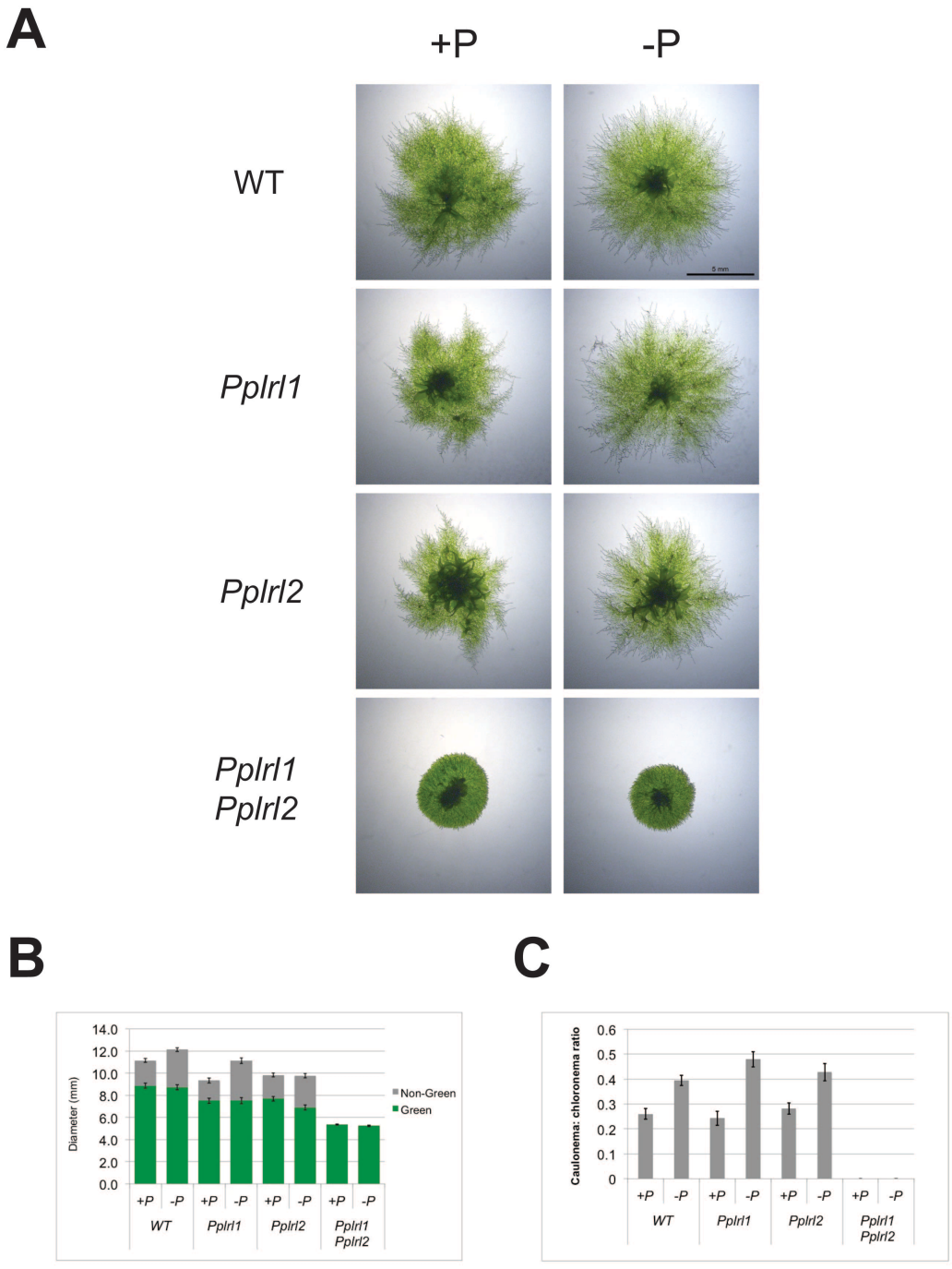


Figure 3-8 A. Effect of low phosphate on protonema development in *Pplrl* loss-of-function mutants. B. Quantification of the green and non-green diameter. C. Quantification of the caulonema : chloronema ratio. Error bars represent SE. The average of 16-20 colonies was taken, except for *Pplrl1 Pplrl2* –P, where 12 were used. Note that *Pplrl1 Pplrl2* double mutants do not develop any caulonema under +P or –P conditions.

3.4 Discussion

Auxin positively regulates rhizoid and caulonema development in *P. patens*. In this chapter I show that *PpLRL* genes mediate auxin response in rhizoids and caulonema in *P. patens.* Results from experiments using exogenous auxin and auxin biosynthesis mutants (*Ppshi1, OXSHI1-5, Ppshi2)* are generally consistent with the hypothesis that auxin positively regulates the expression of *PpLRL* genes. Since *PpLRL* genes positively regulates caulonema and rhizoid development, this result suggests that auxin may induce caulonema and rhizoid development by up-regulating *PpLRL* genes. However, auxin signaling is known to be a complex process (Parry *et al.*, 2009; Del Bianco & Kepinski, 2011). In addition to exogenous auxin level, the control of local auxin biosynthesis, the influx and efflux of auxin, the expression and localization of the auxin signaling molecules such as TIR/AFB auxin receptors, Aux/IAA and ARFs are likely to play a role. The fact that the steady-state transcript levels of *PpLRL* genes are increased in the *Ppiaa1A* mutant highlights this complexity. Interestingly, the *Ppiaa1A* mutant has defective caulonema development despite increased steady-state transcript levels of *PpLRL1* and *PpLRL2*. This result suggested that there are other components may regulate caulonema and rhizoid development. One such component is likely to be *PpRSL3*, a Class II *RSL* gene (Pires *et al.*, 2013). In contrast to the *PpLRL* genes, steady-state level of *PpRSL3* is decreased by more than 100 fold in the *Ppiaa1A* mutants. Since *PpRSL3* is a positive regulator of caulonema development, the decreased in *PpRSL3* expression in the *Ppiaa1A* mutant may explain the defective caulonema development phenotype observed in the *Ppiaa1A* mutants (Prigge *et al.*, 2010). Interestingly, my preliminary transcriptome analysis (**Appendix A3**) show that the expression level of *PpRSL3* is 40-fold lower in the *Pplrl1 Pplrl2* double mutants

than wild type, suggesting that the *PpLRL* genes positively regulates the expression of *PpRSL3*. The emerging picture is therefore a complex gene regulatory network that involves auxin, *PpIAA1A*, *PpLRL* genes, and *PpRSL3* (**Figure**). The fact that auxin also regulates caulonema development via the Class I *RSL* genes *PpRSL1* and *PpRSL2* (Jang & Dolan, 2011; Jang *et al.*, 2011; Pires *et al.*, 2013) adds to the complexity of this network.

My results show that low-phosphate-induced chloronema-to-caulonema transition also requires *PpLRL* gene function. While the low phosphate response is similar to wild type in the *Pplrl2* single mutants, *Pplrl1* single mutants appear to have an enhanced low-phosphate response. This suggests that *PpLRL1* is a negative regulator of low-phosphate response in the protonema, inhibiting the chloronema-to-caulonema transition under low phosphate. However, low phosphate was not able to induce caulonema development in the *Pplrl1 Pplrl2* double mutants. Consistent with this, my preliminary transcriptome analysis showed that the low phosphate response gene *PpPHO1;1* is 8-fold less abundant in the *Pplrl1 Pplrl2* double mutants than wild type (**Appendix A3**). These results show that *PpLRL* genes mediate the low-phosphate-induced caulonema development, but, as in the case of auxin, may involve a complex signalling network.

My results also demonstrated the functional differences between *PpLRL1* and *PpLRL2* in auxin and low-phosphate response. I show that auxin response is significantly more defective in the *Pplrl2* single mutants than the *Pplrl1* mutants, suggesting that *PpLRL2* have a more important role than *PpLRL1* in modulating caulonema and rhizoid development induced by exogenous hormonal signals. On the other hand, while *Pplrl2* mutants behave similarly to wild type under low

phosphate, *Pplrl1* mutant show a strongly low phosphate response. This indicates that *PpLRL1* plays a more important role in low-phosphate response than *PpLRL2*. Together with results from Chapter 2, these data supports the hypothesis that *PpLRL1* and *PpLRL2* show functional divergence. Determining the downstream targets of *PpLRL1* and *PpLRL2* respectively would help to define the extent of overlapping functions between *PpLRL1* and *PpLRL2*, and may shed light on the mechanism underpinning functional divergence following gene duplication.

Since *PpLRL* genes are common components in auxin and low phosphate response, they may be part of a mechanism that integrates low phosphate and auxin signal to control tip-growing cell development in *P. patens*. One interesting experiment would be to determine whether low phosphate response is defective in the auxin signalling mutants, and the role of *PpLRL* genes in this interaction. Further characterization of this network will allow a better understanding of how hormonal, environmental and endogenous developmental signals are coordinated in the development of tip-growing rooting cell development in *P. patens*. The next chapter describes the characterization of the interactions between the *LRL* genes and the Class I *RSL* genes in *P. patens* and *A. thaliana* (Jang & Dolan, 2011; Jang *et al.*, 2011; Pires *et al.*, 2013).

4 Transcriptional interactions between *LRL* genes and the Class I *RSL* genes have changed during land plant evolution

4.1 Introduction

Morphological evolution can be the result of changes in developmental gene regulatory networks (GRNs) (Davidson & Erwin, 2006; de Bruijn *et al.*, 2012). Changes in the wiring of the network (i.e. transcriptional regulations between individual genetic components), including intercalation of extra tiers of transcriptional regulation, and the addition or loss of genes, or their *cis*-regulatory elements, are potential driving forces for morphological evolution (Booth *et al.*, 2010; Soyer, 2010). While a developmental GRN can be assembled *de novo*, there are evidence that existing GRNs can be co-opted into a new context and perform novel functions, as in the co-option of the eye network to control wing pigmentation in butterflies, and the co-option of the KNOX/ ARP network from the shoot apical meristem to the regulation of leaf shape (Monteiro & Podlaha, 2009; Rosin & Kramer, 2009).

In previous chapters, I showed that *LRL* genes are positive regulators of rhizoid and caulonema development in *P. patens* and it is known that *LRL* genes control root hair development in *A. thaliana*, *Oryza sativa,* and *Lotus japonicus* (Ding *et al.*, 2009; Karas *et al.*, 2009; Bruex *et al.*, 2012). The *LRL* genes are thus conserved regulators of tip-growing rooting cell development in land plants. The Class I *RSL* genes are another group of bHLH transcription factors that has also been shown to control both root hair development in *A. thaliana* and rhizoid and caulonema development in *P. patens* (Menand *et al.*, 2007b; Jang & Dolan, 2011; Jang *et al.*,

2011; Pires *et al.*, 2013). Because both *LRL* genes and the Class I *RSL* genes appear to be conserved regulators of tip-growing cell development in land plants, I hypothesized that they may represent a conserved gene regulatory network that controls tip-growing rooting cell development in land plants. In this chapter, I characterized the transcriptional interactions between *LRL* genes and *RSL* genes in *P. patens* and *A. thaliana*, respectively.

4.2 Materials and methods

4.2.1 Plant material

The *Columbia-0* ecotype of *Arabidopsis thaliana* is used unless otherwise stated. Dr. Krzysztof Szczyglowski kindly provided the *AtLRL1+/-* Atlrl*2*, *Atlrl2 Atlrl3* and *AtLRL1+/- Atlrl2 Atlrl3* lines (Karas *et al.*, 2009). The *Atrhd6 Atrsl1* line used in this study is as described previously (Menand *et al.*, 2007b).

4.2.2 Growth conditions for *A. thaliana*

A. thaliana seeds were treated with a sterilization solution (1% sodium hypochloride, 0.05% Triton X-100) then washed at least 3 times in sterile distilled water. The seeds were then sown on sterile Paul's media containing Murashige & Skoog medium, 1% sucrose, and 0.5% Phytagel (Sigma, UK) at pH 5.8 in square petri dishes. To synchronize germination, seeds were stratified on media at 4˚C in the dark for 3-5 days. Plants were grown vertically in a SANYO MLR-351 growth chamber at 25˚C and a 16 h light / 8 h dark regime (with a photosynthesis photon flux density of approximately 40 mol m^{-2} s^{-1} according to manufacturer's manual). Phenotypes of the *Atrhd6 Atrsl1* mutants and the *AtLRL1+/- Atlrl2 Atlrl3* mutants under this condition are as described previously (Menand *et al.*, 2007b; Karas *et al.*, 2009).

4.2.3 RNA extraction, cDNA synthesis and Quantitative RT-PCR analysis

RNA extraction, cDNA synthesis and qRT-PCR in *P. patens* are as described in Chapter 3. For *A. thaliana*, total RNA was extracted from frozen root tissues with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instruction. The RNA is then quantified with a NanoDrop ND1000 spectrometer (Thermo Scientific) and the same amount of RNA from all samples was treated with Turbo DNAse (Ambion, Life Technologies) and used for first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies) using oligo(dT).

The DNAse-treated total RNA was diluted 6.5-fold and used for qRT-PCR analysis directly with SYBR Green PCR Master Mix (Applied Biosystems) and the Applied Biosystems 7300 Real Time PCR system. Three technical replicates were used for each sample. The cycling conditions were as follows: 50˚C 2 min; 95˚C 10 min; then 40 cycles of 95˚C 15 sec, 60˚C 1 min. Dissociation curve analyses were performed at the end of the cycles to ensure the formation of only a single amplicon for each replicate. Baseline and Ct threshold was determined automatically using the bundled software with the Real Time PCR machine. Fold change was calculated with the delta-delta-Ct method in Microsoft Excel. Two reference genes were used for each 96-well plate: *AtPDF2* (At1g13320) (Czechowski, 2005) and an ubiquitin conjugation factor protein (At5g15400). Primers for qRT-PCR was designed to span exon-exon junctions with Primer-BLAST (Ye *et al.*, 2012) then tested with dissociation curve analysis. List of all qRT-PCR primers used is given in **Appendix A5**.

4.2.4 Imaging and microscopy

Stereomicroscopy images of the *A. thaliana* roots were captured with a Leica DFC310 FX camera mounted on a Leica M165 FC stereomicroscope.

4.3 Results

4.3.1 *LRL* genes and Class I *RSL* genes are transcriptionally independent of each other in *P. patens*

Two Class I *RSL* genes, *PpRSL1* and *PpRSL2*, are present in *P. patens* and they positively regulate rhizoid and caulonema development (Menand *et al.*, 2007b; Jang & Dolan, 2011; Jang *et al.*, 2011). Because the *PpLRL* genes also positively caulonema and rhizoid development, I hypothesized that *PpRSL1* and *PpRSL2* may belong to the same genetic pathway as the *PpLRL* genes. To test this, I determined the steady-state transcript levels of the *PpLRL* genes in the *Pprsl1 Pprsl2* mutant background. My results show that there is no different in the steady-state transcript level of *PpLRL* genes in wild type and the *Pprsl1 Pprsl2* background, suggesting that *PpRSL1* and *PpRSL2* do not transcriptionally regulate the expression of *PpLRL* genes (**Figure 4-1**). To determine if *PpLRL* genes control the expression of *PpRSL1* and *PpRSL2*, I determined steady state levels of *PpRSL1* and *PpRSL2* in the *Pplrl1 Pplrl2* double mutant background. No difference was detected in the steady-state transcript level of *PpRSL1* and *PpRSL2* between wild type and the *Pplrl1 Pplrl2* double mutant (**Figure 4-2**). Together, these results showed that the *PpRSL* genes and the *PpLRL* genes are transcriptionally independent of each other. My preliminary transcriptome analysis also supports this conclusion (**Appendix A3**).

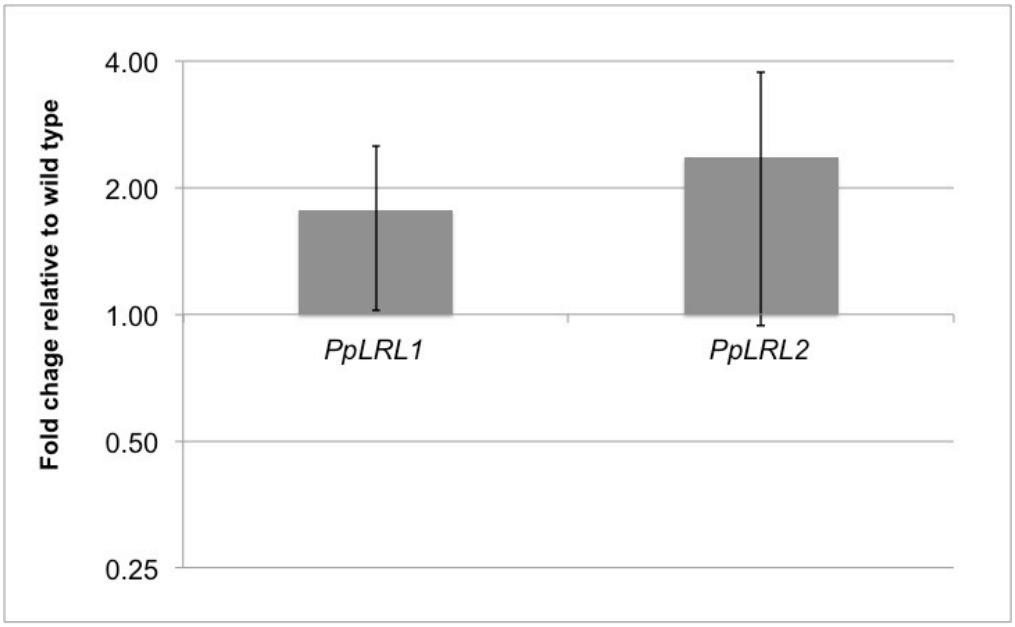


Figure 4-1: Expression of *PpLRL* genes is not affected in *Pprsl1 Pprsl2* mutants. Error bar represents SD.

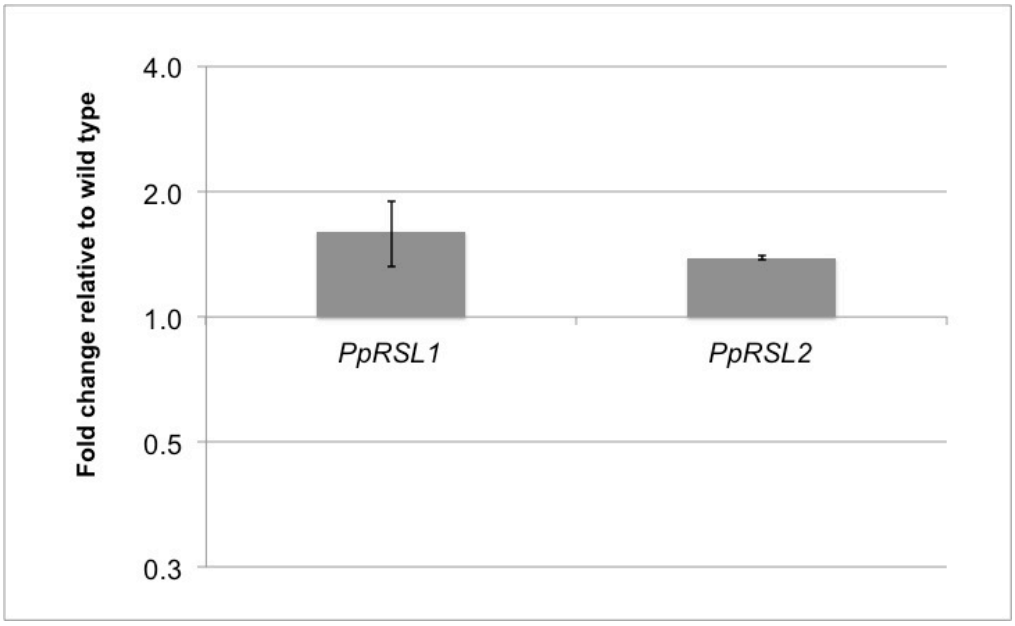


Figure 4-2 Expression of *PpRSL* genes is not affected in *Pplrl1 Pplrl2* background. Error bar represents SD.

4.3.2 Class I RSL genes positively regulates the expression of AtLRL3

The Class I *RHD-SIX-LIKE (RSL)* genes *AtRHD6* and *AtRSL1* are important regulators of root hair development in *A. thaliana* (Menand *et al.*, 2007b). They belong to sub-family VIIIc(1) of plant bHLH transcription factors (Pires & Dolan, 2010).

To determine whether Class I *RSL* genes control the expression of the *LRL* genes in the root, I determined the steady-state transcript level of *AtLRL* genes in the *Atrhd6 Atrsl1* double mutants by qRT-PCR (**Figure 4-3**). Because LRL proteins have diversified in the angiosperms (Chapter 2), it was necessary to determine the interaction of all *AtLRL* genes with Class I *AtRSL* genes.

My result shows that the steady-state transcript levels of *AtLRL3* is down by more than 500 fold in the *Atrhd6 Atrsl1* double mutants compared to WT, but the transcript levels of *AtLRL1, AtLRL2*, *AtLRL4*, and *AtLRL5* do not show significant difference (**Figure 4-3**). These results suggest that *AtRHD6* and *AtRSL1* do not transcriptionally regulate *AtLRL1, AtLRL2, AtLRL4, and AtLRL5*, but positively regulate the expression of *AtLRL3.* This also suggests that the root hair defective phenotypes observed in *Atrhd6 Atrsl1* mutants (Menand *et al.*, 2007b) are due at least in part to the down-regulation of *AtLRL3.*

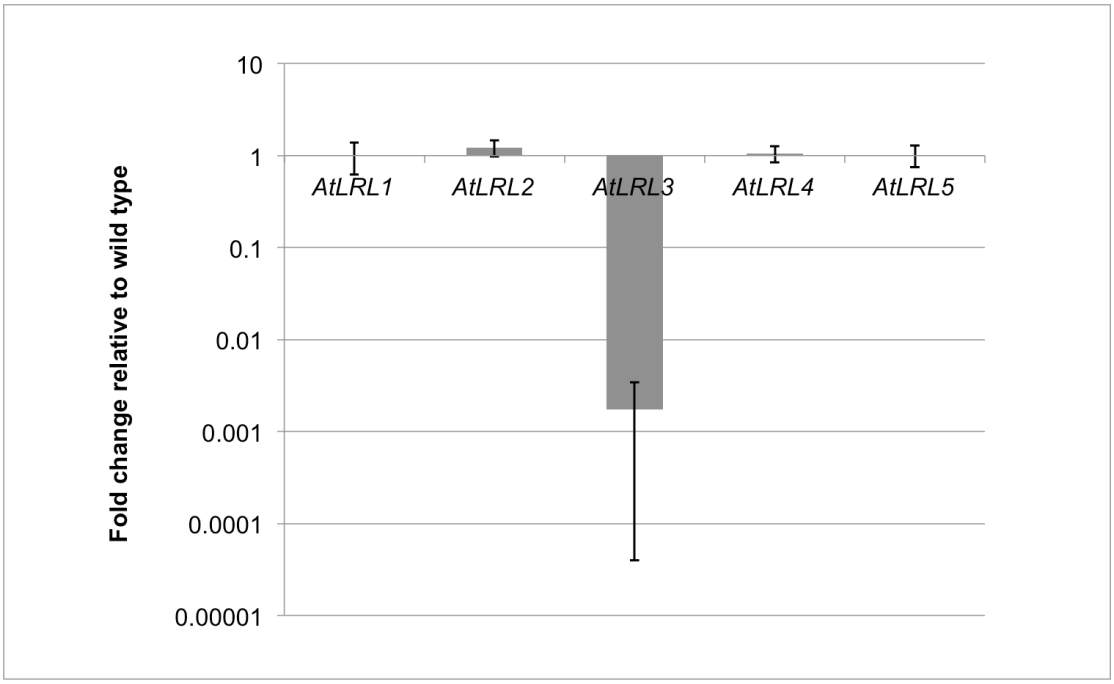


Figure 4-3 Relative expression level of *AtLRL* genes in *Atrhd6 Atrsl1* background. Error bars represent SD.

4.3.3 *AtLRL1*, *AtLRL2*, and *AtLRL3* do not regulate the expression of Class I *RSL* genes

The above experiments show that *AtLRL1* and *AtLRL2* regulate root hair development but they are not regulated by the Class I *RSL* genes. To determine whether *AtLRL1* and *AtLRL2* regulate the expression of Class I *RSL* genes, I determined the steady-state transcript level of *AtRHD6* and *AtRSL1* (the Class I *RSL* genes) in the *Atlrl±1 Atlrl2 Atlrl3* mutants (**Figure 4-4**). My results show that the steady-state transcript level of *AtRHD6* and *AtRSL1* do not show significant difference in the *Atlrl±1 Atlrl2 Atlrl3* mutants compared to WT. This suggests *AtLRL1*, *AtLRL2*, and *AtLRL3* do not regulate the expression of *AtRHD6* and *AtRSL1*.

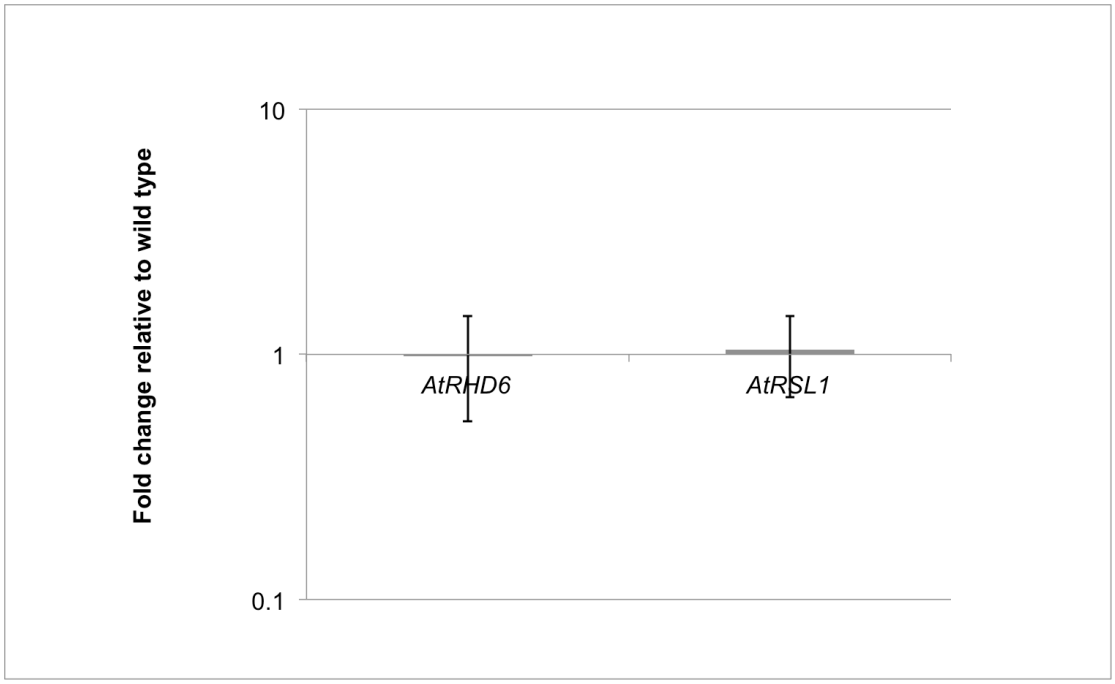


Figure 4-4 the relative expression of *AtRSL* genes in *Atlrl±1/2/3* seedlings.

4.4 Discussion

In this chapter, I characterized the transcriptional regulations between *LRL* genes and Class I *RSL* genes in *P. patens* and *A. thaliana*. My results indicate that that there are no transcriptional interactions between *LRL* genes and Class I *RSL* in *P. patens*, but in *A. thaliana* Class I *RSL* genes positively regulate the expression of *AtLRL3* (**Figure 4-5**). This suggests that a new transcriptional regulation between the *LRL* genes and the Class I *RSL* genes has evolved (**Figure 4-5, Figure 4-6)**. Since changes in the topology of gene regulatory networks can contribute to morphological evolution (Davidson & Erwin, 2006; de Bruijn *et al.*, 2012), this change in transcriptional interaction between *LRL* genes and Class I *RSL* genes could have led to new regulation of root hair growth. In yeast, for example, intercalation of a extra tier of transcriptional regulation led to the intercalation of starvation signal into the mating decision process (Booth *et al.*, 2010). Since the *LRL* genes (Chapter 3) and the Class I *RSL* genes (Jang & Dolan, 2011; Jang *et al.*, 2011; Pires *et al.*, 2013) are also involved in auxin and low phosphate response in tip-growing rooting cells, changes in the topology of this network could lead to changes in how hormones and environmental signals modulate tip-growing rooting cell development in land plants.

The Class II *RSL* genes are another group of transcription factors that interacts with auxin signalling and the Class I *RSL* genes to regulate root hair development in *A. thaliana* (Yi *et al.*, 2010) and caulonema development in *P. patens,* respectively (Pires *et al.*, 2013). My preliminary RNAseq data (**Appendix A3**) suggests that *LRL* genes also interact with one Class II *RSL* gene (*PpRSL3*) in *P. patens* caulonemata. *PpRSL3* expression is regulated by auxin and it is a positive regulator of caulonema development. Together, these data suggest that there are at least three groups of

conserved genetic components (*LRL* genes , Class I *RSL* genes , and Class II *RSL* genes) in the genetic regulatory network underpinning tip-growing cell development in land plants. Further characterization of the interactions between auxin, low phosphate and the *LRL* – Class I *RSL* – Class II *RSL* gene network, in *A. thaliana* and other phylogenetically informative taxa should shed light on how the mechanism underlying the development of tip-growing rooting cell development has evolved during the evolution of land plants.

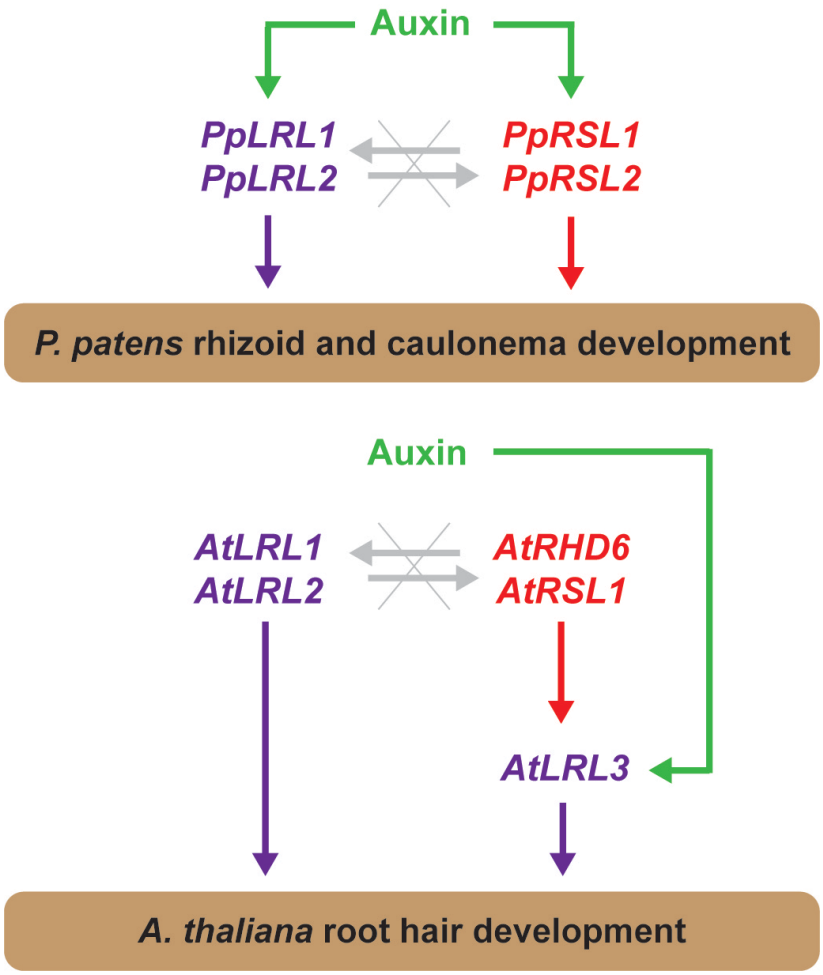


Figure 4-5 A Comparison of the auxin-*LRL*-Class I *RSL* gene network between *P. patens* and *A. thaliana*. In *P. patens*, *LRL* genes and Class I *RSL* genes appear to regulate rhizoid and caulonema development independently. In *A. thaliana*, one *LRL* homolog, *AtLRL3*, is transcriptionally downstream of the Class I *RSL* genes (*AtRHD6* and *AtRSL1*). Auxin, *LRL* genes, and Class I *RSL* genes are in green, violet, and red respectively. Note that some known components associated with this network, such as the Class II *RSL* genes (see text) are omitted from this diagram as their interaction with the *LRL* genes are less well understood. Arrows indicate positive transcriptional regulations. Double opposite grey arrows with a cross indicates no transcriptional interactions. Data from this thesis and (Menand *et al.*, 2007b; Karas *et al.*, 2009; Jang & Dolan, 2011; Jang *et al.*, 2011; Bruex *et al.*, 2012; Pires *et al.*, 2013).

5 Conclusion and general discussion

Tip-growing rooting cells such as root hairs and rhizoids are present at the interface between plant and soil and are important for anchorage, absorption, and interaction with soil microorganisms. They are found in most land plants and are crucial for a terrestrial lifestyle. This thesis aims to understand the genetic basis for the evolution of tip-growing rooting cell in land plants by characterizing the *LRL* genes in *P. patens*. I showed that *LRL* genes are ancient and are likely to be present in the earliest land plants. I showed that *LRL* genes positively regulate rhizoid and caulonema development in the moss *P. patens*. Because *LRL* genes are known to control root hair development in diverse angiosperms, my results thus support the hypothesis that *LRL* genes are conserved regulators of tip-growing cells in land plants. The fact that *LRL* gene function is required for auxin and low phosphate to induce tip-growing rooting cell development in *P. patens* further suggests that *LRL* genes belong to an ancient mechanism that integrates auxin and low phosphate signal to positively control the development of tip-growing rooting cells. One hypothesis for the conservation of multiple components (*LRL* genes, Class I *RSL* genes, and Class II *RSL* genes; Chapter 4) in the mechanism controlling tip-growing rooting cell development is that these components represent a transcriptional gene network that was recruited as a module. However, my results showed that no transcriptional regulations can be found between *LRL* genes and Class I *RSL* genes in either *P. patens* or *A. thaliana*, except that *AtRHD6/AtRSL1* positively regulate *AtLRL3*. This suggested that *LRL* genes and Class I *RSL* genes have gained an transcriptional interaction and the gene network between *LRL* and Class I *RSL* genes have changed since mosses and angiosperms last shared a common ancestor.

As discussed in Chapter 1, one hypothesis for the evolution of the root hairs in the sporophyte of land plants is the recruitment of developmental genes that regulate rhizoid development from the gametophyte (Menand *et al.*, 2007b). Since root hairs and rhizoids perform analogous functions, a wider implication of this hypothesis is that the evolution of a particular character in the sporophyte may be facilitated by the recruitment of genes that control the development of gametophyte structures that performs analogous functions. By showing that the *LRL* genes, which control root hair development in diverse angiosperms (Ding *et al.*, 2009; Karas *et al.*, 2009; Bruex *et al.*, 2012), also control rhizoid development in *P. patens*, my results reinforce this hypothesis and showed that, at least in the evolution of tip-growing rooting cells, recruitment of gametophytic genes play an important role in the evolution of morphological complexity in the sporophyte.

My results presented in this thesis show that the function of *LRL* genes in controlling tip-growing rooting cell development has been conserved in land plants. However, the functions of the *LRL* genes are not limited to the regulation of tip-growing cell development. In *P. patens*, *Pplrl1* mutant develop very short gametophores – a phenotype that is more severe in the *Pplrl1 Pplrl2* mutant – suggesting that both *PpLRL* genes play a role in gametophore development in *P. patens* (data not shown); and in *A. thaliana*, *AtUNE12* (a Class II *LRL*) has been shown to regulate female gametophyte development (Pagnussat *et al.*, 2005). It would be interesting to determine how *LRL* genes diversified functionally during the evolution of land plants.

One of the main tasks of evolutionary developmental biology is to understand how changes in developmental genes or gene networks can lead to changes in

morphology. My thesis contribute to our understanding of plant evolution by showing that the recruitment of pre-existing genetic components from the gametophyte can play a major role in facilitating the evolution of morphological complexity in the sporophyte. Future work that focuses on comparing development mechanisms between diverse land plant taxa within a solid phylogenic framework should lead to a better understanding of the genetic basis for the evolution of tip-growing rooting cells in land plants.

Acknowledgements

Mr. Bruno Catarino (Department of Plant Sciences, University of Oxford), helped with the characterization of the rhizoid phenotypes of *Pplrl*Δ*bL* and *35S:PpLRL-bL* lines (Chapter 2) as part of his Masters project and also helped set up some of the qRT-PCRs described in Chapter 3. I also thank Dr Giulia Morieri, Dr. Holger Breuninger and Dr Nuno Pires for their advice on qRT-PCR.

Appendix

A1 Sequence Alignment used

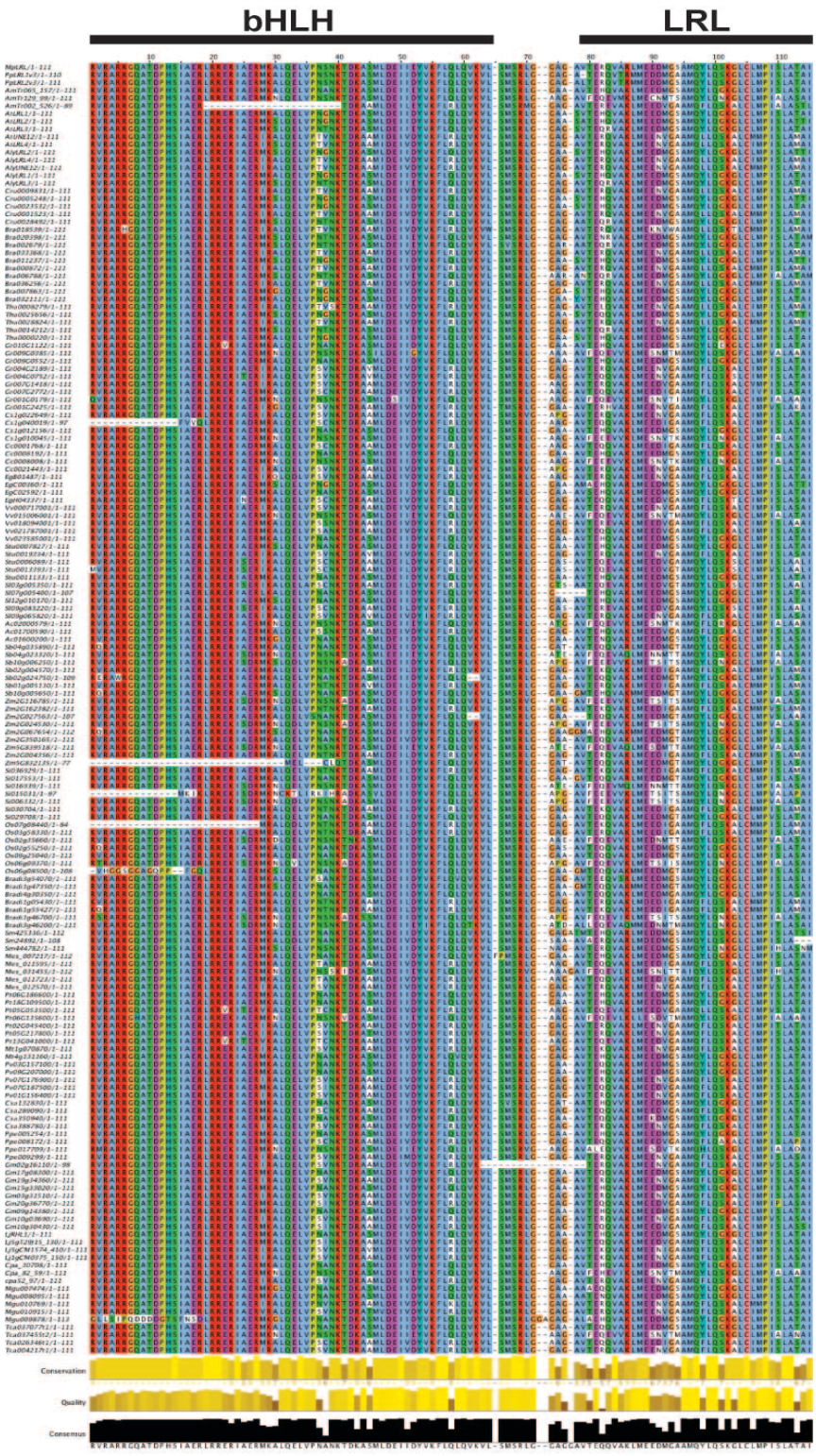


Figure A1-1 Amino acid sequence alignment of land plant LRL proteins. The alignment includes the bHLH domain followed by the LRL domain. Alignments were visualized with Jalview (Waterhouse *et al.*, 2009) with "Clustalx" coloration.

A2 Bayesian and Maximum Likelihood phylogenetic tree for LRL proteins

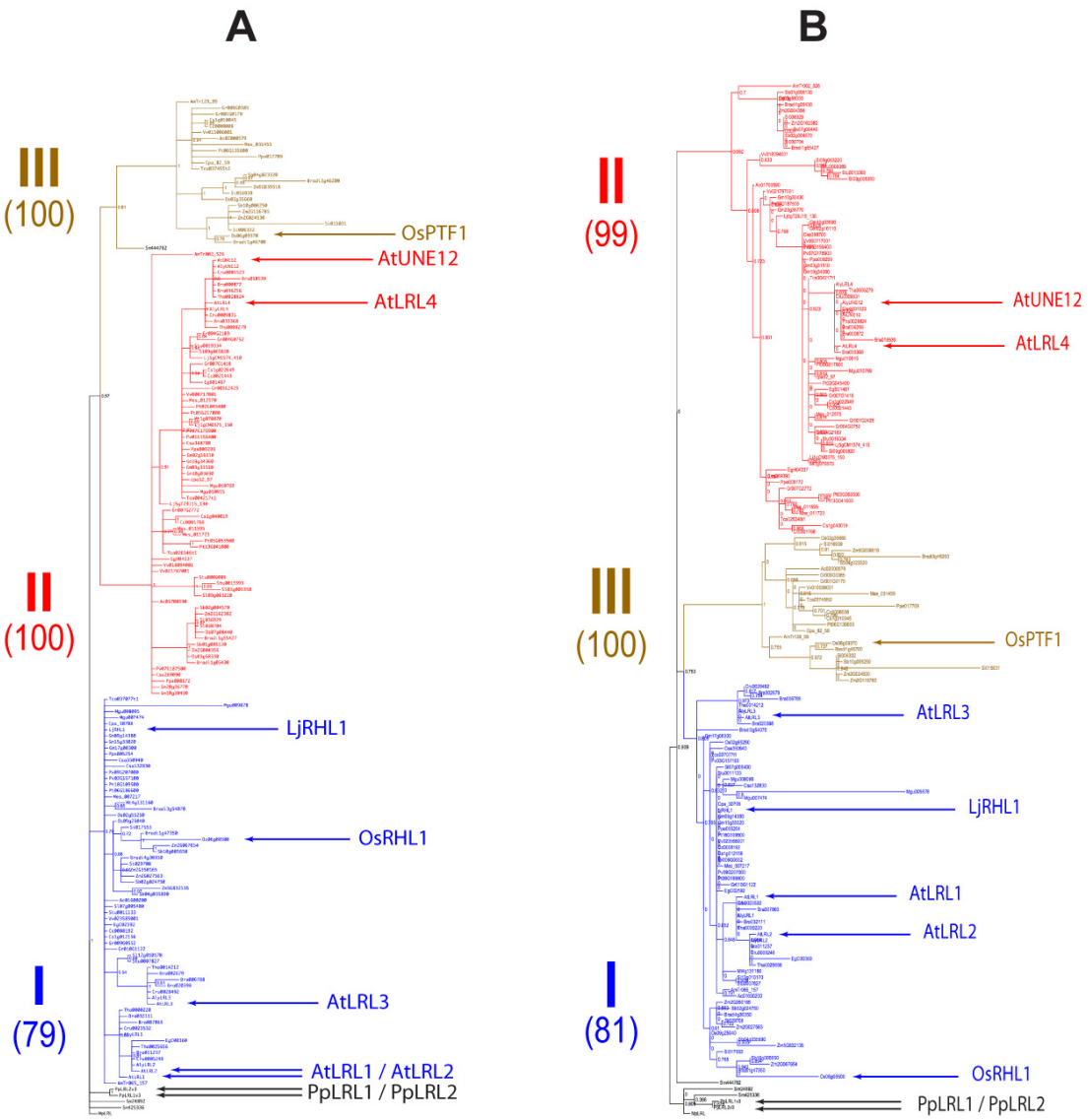


Figure A2-1 Phylogentic analysis of land plant LRL proteins. A: Bayesian tree with posterior probability. B: Maximum Likelihood tree with aLRT. Three clades can be identified in both trees; values in brackets indicate support values (posterior probability and aLRT, for the Bayesian and ML tree, respectively). Note that *PpLRL* genes do not belong to any of the three clades.

A3 Downstream targets of *LRL* genes in *P. patens*

A3.1 Introduction

In the previous chapters, I showed that *PpLRL* genes are positive regulators of tip-growing rooting cell development in *P. patens*. I also demonstrated that *PpLRL* genes act independently of *PpRSL* genes, which also positively regulate rhizoid and caulonema development. To identify genes that are regulated by *PpLRL* and *PpRSL* genes, I used RNAseq to compare the transcriptomes of *Pplrl1 Pplrl2* double mutants and wild type. Due to time constraint a complete analysis of the data is not possible in this thesis. Here I focus on characterizing the biological functions of the downstream targets of *PpLRL* genes using GO-term analyses and utilize this transcriptomic data to independently validate some of the results shown in previous chapters.

A3.2 Materials and methods

A3.2.1 Sample preparation for RNAseq

Macerated protonemata of wild type and *Pplrl1 Pplrl2* double mutants were propagated for 10 days on cellophane discs on basal media. Total RNA was extracted from frozen plant tissue with the MirVana^{TM} miRNA isolation kit (Ambion) then treated with Turbo DNAse (Ambion) to avoid DNA contamination. Two biological replicates were used for each genotype. RNA samples were sent to the Beijing Genome Institute for library constructions and paired-end Illumina sequencing. I thank Mr. Bruno Catarino for helping with the RNA extraction.

A3.2.2 Transcript quantification and differential gene expression analysis

Transcript quantification was performed as described previously (Wang *et al.*, 2013): paired-end reads were subject to quality-based trimming using the FASTX-toolkit [http://hannonlab.cshl.edu/fastx_toolkit/; see also (Goecks *et al.*, 2010)] with a

PHRED quality threshold at 20 and discarding reads <21 nucleotides in length. Transcripts were quantified using RSEM (Li & Dewey, 2011) with default parameters and the predicted coding sequence from gene annotation v1.6 of the *Physcomitrella patens* on Phytozome (Goodstein *et al.*, 2011). Differential gene expression analysis was also performed as described previously (Wang *et al.*, 2013) using DESeq (Anders & Huber, 2010). Differentially expressed genes were identified as those with a Benjamini-Hochberg corrected *P* value ≤ 0.05 (Benjamini & Hochberg, 1995). I thank Dr. Steven Kelly (Department of Plant Sciences, University of Oxford) for performing the transcript quantification and differential gene expression analysis on the raw FASTQ data.

A3.2.3 Enrichment analysis

The enrichment analysis with gene ontology (GO) terms (http://geneontology.org) was performed as described previously (Wang *et al.*, 2013). Significantly enriched annotation terms were identified as those with a Benjamini-Hochberg corrected *P* value ≤ 0.05 (Benjamini & Hochberg, 1995). I thank Dr. Steven Kelly (Department of Plant Sciences, University of Oxford) for performing this enrichment analysis.

A3.2.4 Data visualization with Venn diagram

List of genes from the differential gene expression analysis were fed into VENNY (Oliveros, 2007) for Venn diagrams.

A3.3 Preliminary results

A3.3.1 Biological functions of genes downstream of *PpLRL* genes

To determine the downstream targets of *PpLRL* genes, we compared the transcriptome of *Pplrl1 Pplrl2* and wild type protonema tissues grown on minimal media on top of cellophane discs for one week with RNAseq. We used DESeq (Anders & Huber, 2010) to identify genes that are differentially expressed in the *Pplrl1 Pplrl2* double mutants compared to wild type. Differentially expressed genes are defined here that as those genes that has a Benjamini-Hochberg *P* value ≤0.05. This analysis identified 779 transcripts (including alternative splice forms) that are significantly differentially regulated, of which 616 show lower abundance in the *Pplrl1 Pplrl2* mutants compared to wild type, and 162 that are more highly expressed in the double mutant than wild type.

To categorize the biological function of these differentially expressed genes, we performed GO-term enrichment analyses on these differentially regulated genes. Since I have shown that *PpLRL* genes positively regulate caulonema development in chapter 2, and because caulonema show a higher elongation rate than chloronema cells, I expect that *PpLRL* genes would positively regulate genes that are involved in cell growth processes.

GO-term analysis indicates that genes encoding proteins involved in metal ion transport, cell wall modification and oxidation-reduction processes (A full list is given in **Table A3-1**) are positively regulated by *PpLRL1* and *PpLRL2*; Go-term associated with these processes are enriched among the transcripts that are less abundant in *Pplrl1 Pplrl2* mutant than wild type. Interestingly, GO-terms associated with oxidation-reduction processes are also enriched in transcripts that are more abundant

in *Pplrl1 Pplrl2* mutants than wild type (**Table A3-1**). Since reactive oxygen species and the active modification of cell walls are known to processes associated with root hair development in *A. thaliana* (Foreman *et al.*, 2003; Carol & Dolan, 2006; Lan *et al.*, 2013), These results are consistent with the fact that *LRL* genes positively regulate root hairs development in *A. thaliana* and caulonema development in *P. patens*.

Genes that are less abundant in *Pplrl1 Pplrl2* double mutant protonema than wild type		
Enriched GO-terms	Description	P-value
GO:0004719	protein-L-isoaspartate (D-aspartate) O-methyltransferase activity	3.05E-08
GO:0004097	catechol oxidase activity	2.32E-05
GO:0005618	cell wall	3.97E-05
GO:0030001	metal ion transport	7.16E-05
GO:0005507	copper ion binding	0.00017
GO:0006073	cellular glucan metabolic process	0.00095
GO:0016762	xyloglucan:xyloglucosyl transferase activity	0.00095
GO:0048046	apoplast	0.00095
GO:0046872	metal ion binding	0.00191
GO:0006464	cellular protein modification process	0.00331
GO:0055114	oxidation-reduction process	0.00556
GO:0004601	peroxidase activity	0.00858
GO:0020037	heme binding	0.00904
GO:0008152	metabolic process	0.01188
GO:0006979	response to oxidative stress	0.01513
GO:0008831	dTDP-4-dehydrorhamnose reductase activity	0.01822
GO:0045226	extracellular polysaccharide biosynthetic process	0.01822
GO:0042545	cell wall modification	0.02562
GO:0016491	oxidoreductase activity	0.02789
GO:0008168	methyltransferase activity	0.02789
GO:0030599	pectinesterase activity	0.02789
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0.03293

Genes that are more abundant in *Pplrl1 Pplrl2* double mutant protonema than wild type		
Enriched GO-terms	Description	p-value
GO:0006801	superoxide metabolic process	5.24E-05
GO:0055114	oxidation-reduction process	0.0353

Table A3-1 GO-term enrichment analysis of potential downstream targets of *PpLRL* genes.

A3.3.2 *PpLRL* genes do not regulate the expression of *PpSHI1* or *PpSHI2*, genes that positively regulate auxin biosynthesis in *P. patens*

In Chapter 3, I showed that the steady-state transcript levels of *PpLRL1* and *PpLRL2* are lower in the *Ppshi2* mutant than wild type, suggesting that *PpSHI2* positively regulates *PpLRL1* and *PpLRL2*, possibly through auxin. To determine if there is a transcriptional feedback loop between *PpLRL* genes and *PpSHI2*, I determined whether the *PpSHI2* is differentially expressed in the *Pplrl1 Pplrl2* mutants compared to wild type. The differential gene expression analysis shows that there is no difference (P>0.95) in the expression level of *PpSHI2* between *Pplrl1 Pplrl2* double mutants and wild type.

Steady-state level of *PpLRL1* or *PpLRL2* are not affected in the *Ppshi1* mutant or the *OXSHI1-5* mutant, suggesting that *PpSHI1* do not regulate the expression of *PpLRL* genes. The differential gene expression analysis shows there is also no difference (P>0.95) in the expression of *PpSHI1* between the *Pplrl1 Pplrl2* double mutants and wild type, suggesting that are *PpLRL* genes do not transcriptionally regulate *PpSHI1*.

A3.3.3 *PpLRL* genes do not regulate the expression of *PpIAA1A/B* or *PpIAA2*

In Chapter 3, I showed that the steady-state level of *PpLRL* genes is higher than wild type in the *Ppiaa1A* gain-of-function mutant. The differential gene expression analysis shows that there is also no difference (P>0.95) in the expression of *PpPpIAA1 (Pp1s184_21V6;* according to v3 of the *P. patens* genome annotation, *PpIAA1A* and *PpIAA1B* are alleles of the same gene) between the *Pplrl1 Pplrl2* double mutants and wild type, suggesting that are *PpLRL* genes do not transcriptionally regulate the expression of *PpIAA1A*. Similarly, there is no significant difference in the expression of *PpIAA2* between *Pplrl1 Pplrl2* double mutants and wild type plants.

A3.3.4 *PpLRL* genes positively regulates *PpRSL3*, a Class II RSL gene that regulates chloronema-to-caulonema transition

Like the *PpLRL* genes, *PpRSL3 (Pp1s60_187V6)* is a positive regulators of caulonema development in *P. patens* (Pires *et al.*, 2013). The differential gene expression analysis showed that *PpRSL3* is approximately 40-fold less abundant in the *Pplrl1 Pplrl2* mutants ($P < 0.001$). This suggests that *PpLRL* genes positively regulate *PpRSL3*. In the *Ppiaa1A* mutant, the steady-state transcript level of *PpRSL3* is decreased by more than 100-fold while the steady-state transcript level of *PpLRL1* and *PpLRL2* are increased by approximately 4 fold. Together, these results suggest that a complex gene regulatory network exists between *PpIAA1A*, *PpLRL* genes and *PpRSL3*.

A3.3.5 *PpLRL* genes positively regulate the expression of *PpPHO1;1*

Low phosphate induces caulonema development in wild type *P. patens* and strongly induce the expression of *PpPHO1;1 (Pp1s353_59V6)* in the protonema (Wang *et al.*, 2008). *PpPHO1;1* is thus a low phosphate response gene in the protonema. In *A. thaliana*, homologs of *PpPHO1;1*, which belongs to the *PHO1* family, are involved in phosphate loading into xylem and the expression of *AtPHO1* is increased during phosphate deficiency (Wang *et al.*, 2004; Stefanovic *et al.*, 2007). In Chapter 3, I showed that the induction of caulonema development by low phosphate requires *PpLRL* gene function. Consistent with the role of *PpLRL* genes in low phosphate response, *PpPHO1;1* is approximately 8-fold less abundant in the *Pplrl1 Pplrl2* mutants ($P < 0.001$). The observation that *PpLRL* genes positively regulate a gene that is induced by low phosphate treatment is consistent with my previous results showing that the low phosphate response in protonema requires *PpLRL* gene function (Chapter 3).

A3.3.6 Transcriptomic data confirms that *PpLRL* genes do not regulate the steady-state transcript level of *PpRSL1* or *PpRSL2*

In Chapter 4, I showed, using qRT-PCR, that the steady-state transcript level of *PpRSL1 (Pp1s147_136V6)* and *PpRSL2 (Pp1s84_14V6)* are not significantly affected in the *Pplrl1 Pplrl2* mutants. This result is confirmed by the differential gene expression analysis, which shows that there is no difference (P>0.95) in the expression levels of *PpRSL1* and *PpRSL2* between *Pplrl1 Pplrl2* mutants and wild type.

A3.4 Acknowledgements

Dr. Steve Kelly (Department of Plant Sciences, University of Oxford) carried out the transcript quantification and differential gene expression and enrichment analyses on the raw RNAseq data, which were generated by GATC Biotech or the Beijing Genome institute. Mr. Bruno Catarino helped with some of the RNA extractions.

A4 *PpCCD8* does not regulate the expression of *PpLRL* genes

Protonema development is controlled by strigolactones. Mutants defective in the moss homolog of strigolactone biosynthesis gene *PpCCD8* show increased protonema branching and this phenotype can be partially rescued by addition of the strigolactone analog GR24 into the media (Proust *et al.*, 2011). Based on these results, strigolactones have been proposed to be a quorum-sensing signal and above-threshold level of strigolactone may serve to limit colony extension (Proust *et al.*, 2011). Interestingly, *PpCCD8* are not expressed in the protonema filaments but are expressed at the base of the gametophores (Proust *et al.*, 2011), suggesting that the effect of strigolactones on protonema development may be due to excreted strigolactones in the media.

In Chapter 2 I show that *PpLRL* genes control colony growth by positively regulating caulonema development. To test if strigolactones control the expression of *PpLRL* I determined the steady state level of *PpLRL* genes in the *Ppccd8* mutants. The steady-state levels of neither PpLRL1 nor PpLRL2 are significantly different from wild type (**Figure A4-1)**, suggesting that *PpLRL* genes are not involved in controlling strigolactone-mediated colony extension.

In conclusion, these preliminary data suggest that strigolactones and *PpLRL* genes control protonema development independently.

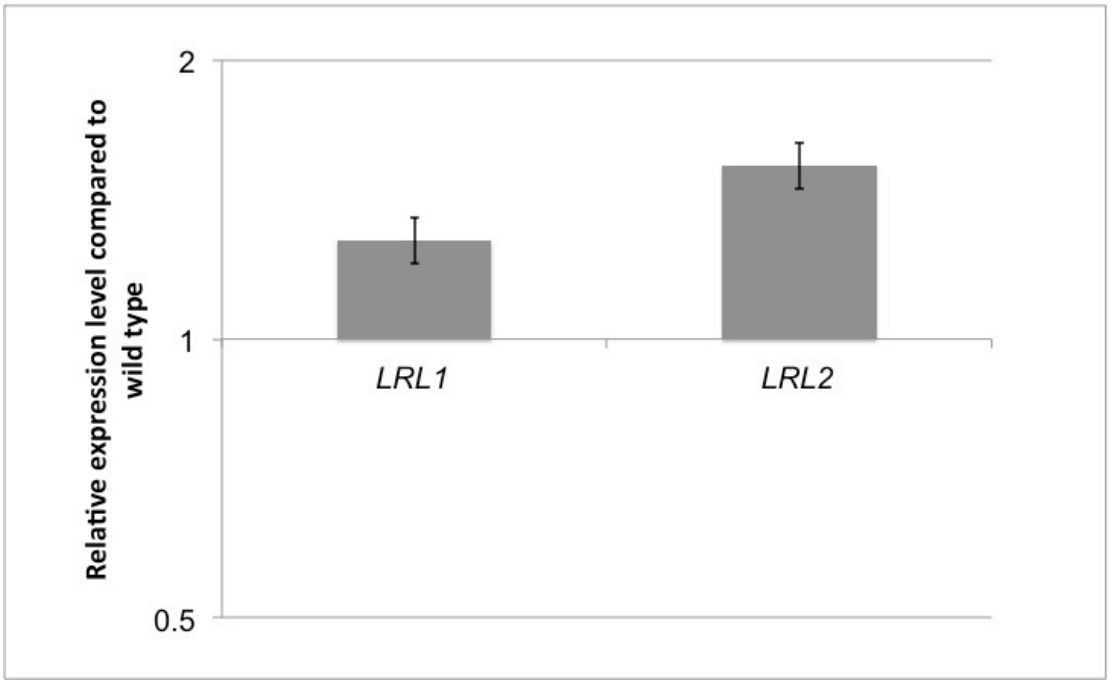


Figure A4-1 Expression of *PpLRL* genes in the strigolactone biosynthesis mutant *Ppccd8*. Error bars represent Standard error of the mean.

A4.1 Acknowledgements

I thank Ms. Beate Hoffmann and Dr. Catherine Rameau's (INRA Centre de Versailles-Grignon, France) for providing the *Ppccd8* lines (Proust *et al.*, 2011).

A5 Lists of primers used

A5.1 For *Physcomitrella patens*

For generating mutant lines

PpLRL1_5'HR_F_SalI	gcgtcgacagagacaccgaccagcaagt
PpLRL1_5'HR_R_BamHI	gcggatccaacctcgaaatccttccaa
PpLRL1_3'HR_F_MluI	cgacgcgtagcactcttcagctcaacacaa
PpLRL1_3'HR_R_NcoI	ggccatggaatttcattccggcgtatg
PpLRL2_5'HR_F_SalI	cggtcgacccgacgatatacgaccaat
PpLRL2_5'HR_R_BamHI	gcggatccttctgacgcaagacaccaa
PpLRL2_3'HR_F_SpeI	gcactagtggccctgatgttcttcatgt
PpLRL2_3'HR_R_AscI	gcggcgcgcctggaagccactttatgtcca
PpLRL1ΔbL_5'HR_F_XbaI	cgtctagacgtatggcgattccttcatc
PpLRL1ΔbL_5'HR_R_BamHI	gcggatccgcaaagcgagaaacttccat
PpLRL1ΔbL_3'HR_F_MluI	cgacgcgtcaggatctgcagctggtgt
PpLRL1ΔbL_3'HR_R_NcoI	ggccatggtgcctaaacaaatgcagtacg
PpLRL2ΔbL_5'HR_F_BamHI	gcggatcctgtatcttgcaggggctacat
PpLRL2ΔbL_5'HR_R_XhoI	ggctcgagctccctgctaaccaagaaaaca
PpLRL2ΔbL_3'HR_F_SpeI	cgactagtgctcatgccaggttctgc
PpLRL2ΔbL_3'HR_R_MluI	cgacgcgtggatatgcctgaacgaatgc
PpLRL1_bHCDS_START_F	atgccacctggaggttgt
PpLRL1_bHCDS_STOP_R	ctaggtgtcttgagtggtagattca
PpLRL2_bHCDS_START_F	atgtcaactggaagtgccca
PpLRL2_bHCDS_STOP_R	ttaggtcccttgagtggcac

For PCR-genotyping of mutant lines

PpLRL1_P1	ggaaggttctgatcggtttg
PpLRL1_P2WT	atacggctgctgaagatgct
PpLRL1_P2hyg	ggtggagctcggtaccataa
PpLRL1_P3	cgtcgtggtcaagctactga
PpLRL1_P4	cactctcccagcctctgaac
PpLRL1_P5WT	atacgtgccctgctactgct
PpLRL1_P5hyg	gccggccagatctataacttc
PpLRL1_P6	ttggtgaagttgtcttccctct
PpLRL2_P1	cacaaggtttgatcgggtct
PpLRL2_P2WT	gaccatcaactggtcgcttt
PpLRL2_P2neo	gaattcgagctcggtaccat
PpLRL2_P3	tggtgcacttccttcacttg
PpLRL2_P4	gcagtctctgaaccgtcctc
PpLRL2_P5WT	gctctgatgtgcgacttaacc
PpLRL2_P5neo	gccggccagatctataacttc
PpLRL2_P6	ccccattgtcaattctacctg

For qRT-PCR

PpGAPDHq1_F	cttgagaagcctgcctccta
PpGAPDHq1_R	tgctgtcggtaatgaagtcg
PpEF1a	ggatcttgtcggggttgtaa
PpEF1a	tttcaccttgggagtgaagc
PpRSL1_F	gtgtcctattccgaggacca
PpRSL1_R	caaccttgaggtcccaaaaa
PpRSL2_F	aggataagtgagcggctgaa
PpRSL2_R	ccttgggccagatagaatca
PpLRL1_CDS_F3	cgtggtcaagctactgatcct
PpLRL1_CDS_R3	cgccttatccgtcttattcg
PpLRL2_CDS_F2	agtattgctgaacggcttcg
PpLRL2_CDS_R2	cagcacctcctaatcgactca
PpLRL1_q3F	ccatctcccttgcaactgct
PpLRL1_q3R	tggaaccagctgtggacttg
PpLRL2_q3F	aagggtctgggtatgccaac
PpLRL2_q3R	ctgtgcggatcagttgcttg

For generating the GUS-reporter lines

PpLRL1GUS_5'HR_nF1_AscI	taggcgcgccgaaggttctgatcggtttgg
PpLRL1GUS_5'HR_nR1_NcoI	ggccatggcgcttccactagacaacctc
PpLRL1GUS_3UTR_nF1_BamHI	gcggatccaagacacctaggagctcatgc
PpLRL1GUS_3UTR_nR1_BamHI	gcggatccttgtgttgagctgaagagtgc
PpLRL2GUS_5'HR_nF1_AscI	gcggcgcgccacaaggtttgatcgggtct
PpLRL2GUS_5'HR_nR1_NcoI	ggccatggcgctccctctccacgact
PpLRL2GUS_3UTR_nF1_BamHI	caggatccgtgccactcaagggacctaa
PpLRL2GUS_3UTR_nR1_HindIII	gacaagcttgaagaacgaacaagtgctcca

For PCR-genotyping of the GUS-reporter lines

PpLRL1_P7	acggggagaggaaggttct
PpLRL1_P8WT	atacggctgctgaagatgct
PpLRL1_P8GUS	caacgctgatcaattccaca
PpLRL1_P10WT	aagtgacacgcatgatggag
PpLRL1_P10GUS	ggtggagctcggtaccataa
PpLRL1_P11	ttggtgaagttgtcttccctct
PpLRL2_P7	agtttacaaagccccgaggt
PpLRL2_P8WT	gaccatcaactggtcgcttt
PpLRL2_P8GUS	caacgctgatcaattccaca
PpLRL2_P10GUS	ggtggagctcggtaccataa
PpLRL2_P11	cacgccaagatgtgtgagtt

For Southern blots

HYGprobe-F	atccggtcggcatctactct
HYGprobe-R	tgtaggagggcgtggatatg
NEOprobe-F	tgaatgaactgcaggacgag
NEOprobe-R	aatatcacgggtagccaacg

A5.2 For Arabidopsis thaliana

For qRT-PCR

At5g15400_F	TGCGCTGCCAGATAATACACTATT
At5g15400_R	TGCTGCCCAACATCAGGTT
AtPDF2_q1F	TAACGTGGCCAAAATGATGC
AtPDF2_q1R	GTTCTCCACAACCGCTTGGT
AtLRL1_qF1	AAGAACTCGTTCCTAACGGC
AtLRL1_qR1	TGCTCATGCTCAGTACCTTG
AtLRL2_qF2	TCAAATCTCTGAGGATGCCG
AtLRL2_qR2	GCTGATCCCATGTCCTCTTC
AtLRL3_qF3	CCAACAAGACGGATAAGGCA
AtLRL3_qR3	TCTGCGGAGAGACCATTGA
AtLRL4_qF1	GGCTCCAAGTTAAGGTCCTG
AtLRL4_qR1	CCTCTCTGTCCCATCGTTTG
AtLRL5_qF1	AGTTGAGGATGAAACGGGTG
AtLRL5_qR1	GATTGAAGAAGCTGCATCGC
AtRHD6qPCR_U	CCTAAATCCGCTGGAAACAA
AtRHD6qPCR_L	CTCTTCGATTCTTGGCTGCT
AtRSL1qPCR_U	CCCTAAACTGGCTGGCAATA
AtRSL1qPCR_L	TCTTGGCTGCTAGGCTTTGT

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