Functional Analysis of Class 1 RSL Genes in Caulonema and Rhizoid Differentiation of Physcomitrella patens

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>bHLH</td>
<td>Basic helix loop helix</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Disodium Edetate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GUS</td>
<td>Beta-glucuronidase</td>
</tr>
<tr>
<td>H cell</td>
<td>Root Hair-forming cell</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N cell</td>
<td>Non hair-forming cell</td>
</tr>
<tr>
<td>NAA</td>
<td>α-naphthaleneacetic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NPA</td>
<td>Naphthylphthalamic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TIBA</td>
<td>2,3,5-triiodobenzoic acid</td>
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<tr>
<td>WT</td>
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Abstract

Caulonema and rhizoids are tip growing filamentous cells required for nutrient acquisition and attachment to substratum in Physcomitrella patens (P. patens). Caulonema differentiate from developing protonema while rhizoids differentiate from gametophores. Even though these two filamentous cells differentiate from different haploid stages of moss development, both caulonema and rhizoid differentiation are regulated by class 1 RSL genes (PpRSL1 and PpRSL2) and auxin; mutant plants which lack both PpRSL1 and PpRSL2 functions suppress caulonema and rhizoid differentiation, whereas auxin treatment enhances caulonema and rhizoid differentiation in moss development. However, the regulatory relationship between class 1 RSL and auxin is previously unknown in caulonema and rhizoid differentiation. This study shows that auxin regulates caulonema and rhizoid differentiation by positively regulating PpRSL1 and PpRSL2 expression in protonema and gametophore development; auxin treatment increases the transcriptional expression levels of PpRSL1 and PpRSL2 and mutant plants that lack both PpRSL1 and PpRSL2 do not differentiate caulonema and rhizoids even in auxin-treated conditions. Furthermore, co-overexpression of PpRSL1 and PpRSL2 enhances caulonema and rhizoid differentiation in the absence of auxin. These findings suggest that the regulation of class 1 RSL genes by auxin controls caulonema and rhizoid differentiation in moss development.

In contrast to P. patens, class 1 RSL genes regulate root hair differentiation independently of auxin in Arabidopsis thaliana (A. thaliana). These observations suggest that the regulatory interaction between class 1 RSL genes and auxin has changed during land plant evolution.
Chapter One:
Introduction
1.1 The life cycle of *Physcomitrella patens*

*Physcomitrella patens*, a member of the bryophyte grade of land plants, is used as a model system in plant sciences (Cove and Knight, 1993). *P. patens* has several advantages as a model plant. The gene targeting technique (by homologous recombination) makes it relatively easy to generate mutants and to analyze their function (Schaefer and Zríäd, 1997; Hiwatashi et al., 2001; Hohe et al., 2004; Kamisugi et al., 2005); F2 progeny and test crosses are not required (Cove, 2005). The anatomically simple structure is another advantage. Mosses protonema comprises three major kinds of cells, chloronema, caulonema and rhizoids. The simplicity in morphology makes it easy to study cell differentiation and development.

Recently, phylogenetic analysis indicates that mosses constitute the second diverging clade of land plants and is therefore representative of one of the earliest groups of land plants (Shaw and Renzaglia, 2004; Renzaglia et al., 2007). In these respects, *P. patens* is a good model plant for studying the evolutionary basis of plant cell differentiation and development.

The life cycle of *P. patens* exhibits alternation of generations, in which there is a multicellular haploid gametophyte and a multicellular diploid sporophyte (figure 1.1). The gametophyte consists of two distinct developmental stages, protonema and gametophore. The protonema is a filamentous network of haploid cells and develops from germinating haploid spores and gametophore is a haploid leafy shoot system which develops from the bud. Buds are three-faced apical cells which form on protonema filaments and function as initials of gametophores. The diploid sporophyte develops from mature gametophores bearing the sexual organs, archegonia and antheridia. After fertilization, a zygote develops into diploid sporophyte for haploid spore production.
Figure 1.1 The life cycle of *P. patens*.
1.1.1 Protonema development

Protonema, a developmental stage of haploid filamentous cells, develops from germinating spores. The protonemal filaments comprise two different types of cells, chloronema and caulonema. The first cells to develop from the spore are chloronema (Cove and Knight, 1993). Chloronema cells are short and contain numerous chloroplasts and perpendicular cell walls, compared to caulonema. The numerous well-developed chloroplasts are very similar to those of leaf cells in ultrastructure, suggesting that the main function of chloronema cells in protonema development is photosynthetic assimilation (Tewinckel and Volkman, 1987; Duckett et al., 1998). Chloronemal filaments grow at their tips by new chloronema cells being added by apical chloronemal cell division and tip growth (Cove, 2005; Menand, 2007a); chloronema cell division occurs about every 24 hours and the tip growth rate is approximately 7 µm/h (Duckett et al., 1998; Schween et al., 2003; Menand et al., 2007a). As chloronemal filaments elongates, apical chloronema cells develop into caulonema in the growing edges of colonies. Caulonema cells are relatively long and contain few chloroplasts and oblique cross cell walls. The morphological characteristics of caulonema cells are very similar to those of rhizoids which form from gametophores. Caulonema are hypothesized as specialized cells which are involved in nutrient uptake and transport and it is likely that rhizoids may play a similar function in nutrient uptake as well as playing a role in anchorage; caulonema acquire nutrients and transport them back to the proximal chloronema cells which perform a high rate of photosynthesis (Duckett et al., 1998; Pressel, et al., 2008). Caulonema cells grow at their tips by apical cell division and tip growth, but cell division and growth rates of caulonema cells are much faster than those of chloronema; the growth rate of caulonema is around 30 µm/h and the cell division of apical caulonema occurs approximately every 7 hours (Cove and Knight, 1993; Duckett et al., 1998; Reski, 1998; Menand et al., 2007a).
Chloronema and caulonema cells are also different in some cellular organelle shape and location. Nuclei and chloroplasts of chloronema cells are spherical in shape and located peripherally while caulonema cells have relatively elongated nuclei in the centre of caulonema cells and few spindle-like chloroplasts along endoplasmic strands. Faster growth rate of caulonema cells than chloronema cells are probably related to the cytological difference between caulonema and chloronema (Pressel, et al., 2008). Another interesting cytological difference between chloronema and caulonema is vacuolar features. The chloronema cell contains a single large vacuole in the centre of cells. Young apical caulonema cells which are newly divided from sub-apical caulonema cells usually contain a single vacuole behind the central nucleus. As the caulonema cells age, the single large vacuole disappears in caulonema cells. Instead, many small vacuoles develop in mature sub-apical caulonema cells. It is hypothesized that the disappearance of single large vacuole is related to the roles of caulonema in nutrient transport. (Duckett et al., 1998; Pressel, et al., 2008).

The developmental transition from chloronema into caulonema is regulated by environmental growth conditions; ammonium tartrate (as a source of nitrogen) activates chloronema development and suppresses caulonema differentiation (Jenkins and Cove, 1983). In contrast, caulonema differentiation is induced in the absence of sufficient nitrogen and phosphate sources. Auxin also affects caulonema development; auxin treatment activates caulonema differentiation while an anti-auxin PCIB ($p$-chlorophenoxyisobutyric acid) treatment suppresses caulonema differentiation in protonema development, suggesting that auxin positively regulates caulonema differentiation from chloronema (Johri and Desai, 1973; Sood and Hackenberg, 1979; Bopp, 1980).
Some genes are involved in the developmental transition from chloronema into caulonema. *PpARPC* encodes the subunit of the actin-related protein2/3 (Arp2/3) complex. The actin-related protein2/3 (Arp2/3) complex regulates actin filament dynamics which play an important role in shaping the actin cytoskeleton (Mullins et al., 1998; Harries et al., 2005; Perroud and Quatrano, 2006). In *P. patens* the *Pparpc1* mutant plants develop few of the rapidly elongating caulonema cells which are found at the growing edge of the wild type (Harries et al., 2005). The mutant phenotype can be mimicked by latrunculin B, an actin depolymerisation drug. In latrunculin B-treated conditions, all cells which develop in protonemal filaments are chloronemal. This result shows the important role of actin cytoskeleton in tip-growing caulonema development. The role of the cytoskeleton in caulonema development was also tested by cremart treatment, a microtubule destabilizing drug (Doonan et al., 1988). Cremart treatment completely destroys microtubule cytoskeleton and causes apical cells to swell without tip growth. These data indicate actin and microtubule cytoskeletons are required for caulonema differentiation in protonema development. Another gene, *PpHXK1* (hexokinase of *P. patens*), regulates caulonema differentiation (Thelander et al., 2005). Mutant plants which lack the hexokinase gene show some defects in caulonema differentiation. Interestingly, the *Pphxk1* mutants also show hypersensitivity to cytokinin and abscisic acid and induce bud formation on chloronemal filaments, which is not observed in wild type. However, both *Pparpc1* and *Pphxk1* mutants also affect the chloronema morphology. This suggests that both the *PpARPC* and *PpHXK1* genes are involved in general cell growth and are likely not to be specifically involved in caulonema differentiation from chloronema.

### 1.1.2 Gametophore development
Gametophores constitute the haploid leafy shoot systems that develop during the moss life cycle. The protonemal buds are the initials of the erect moss developments. Buds develop from side branch initials which usually develop from caulonemal filaments (Schumakerl and Dietrich, 1997; Cove, 2005). After caulonema cell division in the distal region of protonemal filaments, a small swelling usually forms at the apical end of the second or third sub-apical cells and subsequently develops into a side branch initial. Before side branch cell division, bundles of microtubules appear between the nucleus and the prospective division site and the nucleus starts to move to the site in which side branch initials form. The microtubules become gradually thicker and shorter as the nucleus moves to the side branch initial site. The role of microtubule in nuclear movement in side branch initial development was tested by treatment with cremart, a microtubule destabilizing drug and taxol, a microtubule stabilizing drug. Cremart treatment inhibits nucleus movement and concomitant taxol treatment suppresses the inhibition by cremart (Doonan et al., 1986). The daughter nucleus moves into the emerging side branch initial cell and the other moves back to the middle of caulonema. The side branch initial cells develop into buds by asymmetrical, three-dimensional cell division. Apical bud cells having many chloroplasts divide further to form a gametophore stem while basal cells having few chloroplasts divide to develop rhizoids.

The side branch initials can develop into either lateral side protonemal filaments or buds. Determination of side branch initial cell fate is regulated by cytokinin. Cytokinin positively regulates bud formation from the side branch initial cells (Ashton et al., 1979; Reski and Abel, 1985; Schumakerl and Dietrich, 1997). In absence of cytokinin, bud formation is inhibited and side branch initial cells develop into later lateral side filaments by apical cell division and tip growth whereas in cytokinin-treated condition, side branch
initials develop into buds by three-dimensional and asymmetric cell division, suggesting that cytokinin is an essential hormone to control developmental transition from protonema into gametophores.

Gametophores of *P. patens* develop two types of leaves, juvenile leaves and adult leaves. Juvenile leaves develop on the young gametophore axis and adult leaves appear after the formation of several juvenile leaves. A morphological difference between juvenile and adult leaves is the absence and presence of a midrib (Sakakibara et al., 2003). The single cell layer of gametophore leaf is very simple compared to the multi cell layers of angiosperm leaf (Cove et al., 2006). Consequently there are no guard cells, specialized cells for gas exchange do not exist in moss leaves. It is hypothesized that gametophore leaves can perform gas exchange without the aid of guard cells because of simple single cell layer structure.

Rhizoids are specialized rooting cells found in early diverging groups of land plants, such as mosses, liverworts and hornworts. Land plants are anchored to soil from which essential inorganic nutrients are taken up. These functions are carried out by rhizoids in early diverging groups of land plants and by root hairs in vascular plants such as *A. thaliana* (Gahoonia et al., 1997; Duckett et al., 1998). Despite the morphological difference between multi-cellular rhizoids in non vascular plants and single cellular root hairs in vascular plants, rhizoids and root hairs similarly respond to environmental growth factors such as nutrients and hormones; nutrient rich conditions suppress rhizoid and root hair developments (Goode et al., 1992; Ticconi et al., 2001) and exogenous auxin treatment enhances both rhizoid and root hair development (Johri and Desai, 1973; Ashton et al., 1979; Pitts et al., 1998). However, the mechanism of rhizoid development is poorly
understood at both the molecular and genetic level, because of the lack of useful model systems. *P. patens* is a good model system suitable to study rhizoid development because techniques required for transformation and gene targeting mutagenesis have been established (Nishiyama et al., 2000; Schaefer, 2001; Trouiller et al., 2005). Furthermore, the whole genome of *P. patens* has been sequenced (Rensing et al., 2008).

In *P. patens*, rhizoids are multicellular and develop on specialized epidermal cells on the leafy shoot system (gametophores). Rhizoids are very similar in character to caulonema. Rhizoids have poorly developed chloroplasts and brown pigments. They divide at their tips and new rhizoid cells are oriented obliquely across the cells (Reski, 1998). The cytological feature of rhizoid cells is similar to caulonema of protonemal colonies and root hairs (Duckett et al., 1998; Pressel, et al., 2008). There are two kinds of rhizoids in *P. patens*, which can be distinguished by the position on the gametophores; basal rhizoids develop at the epidermal cells below juvenile leaves which do not have a midrib, whereas mid-stem rhizoids develop in more apical regions below adult leaves which have a midrib (Sakakibara et al., 2003). Mid-stem epidermal cells which give rise to mid-stem rhizoids are distinguished from other mid-stem epidermal cells which do not differentiate mid-stem rhizoids by their location in the gametophores; when viewed in transverse section, mid-stem rhizoid-forming cells are located to the outside of two small cells of the leaf trace cells, that extend from the stem into the midrib of the adjoining leaf, whereas other mid-stem epidermal cells are located to the outside of the normal cortex cells.

**1.1.3 Sporophyte development**

The sporophyte is the multicellular diploid stage in the moss life cycle. Male and female gametes are produced in the antheridia and the archegonia of the gametophyte,
respectively. For fertilization, moist conditions are required for the motility of sperm cells (Cove, 2005). The fertilized zygote starts to expand and fill the archegonium cavity. After the expansion, the zygote begins cell division and develops into sporophyte consisting of a seta and a sporangium. The first zygotic cell division produces an apical and a basal cell with asymmetrical cell division (Sakakibara et al., 2008). The apical cell as a stem cell divides obliquely and stops dividing after around 12 cell divisions. New cells which are produced by apical zygotic cell division continue dividing and develop into a sporangium. The role of \( PpLFY1 \) and \( PpLFY2 \) genes in zygotic cell division has been characterised (Tanahashi et al., 2005). \( PpLFY1 \) and \( PpLFY2 \) are homologues of Arabidopsis \( LFY \) genes. The \( LFY \) gene in \( A. \) thaliana is a key regulator of developmental transition from the vegetative to the reproductive stage without controlling cell division or expansion directly (Blázquez and Weigel, 2000). However, the \( LFY \) genes of \( P. \) patens regulate sporophyte development by controlling zygotic cell expansion and division after fertilization; the first zygotic cells of the mutant plants which lack both \( PpLFY1 \) and \( PpLFY2 \) arrest without the cell expansion and division. Furthermore, gametophore morphology of the double mutant are indistinguishable from wild type, suggesting these genes control sporophyte development by specifically regulating zygotic cell development in \( P. \) patens (Tanahashi et al., 2005).

A seta which develops from a seta meristem in the middle of a sporophyte is located between sporangium and the base of sporophyte. Class 1 \( KNOX \) (\( KNOTTED1\)-LIKE \( HOMEOBOX \)) genes of \( P. \) patens were identified and shown to be involved in seta meristem development. Class 1 \( KNOX \) transcription factors regulate shoot apical meristem development by controlling the action of phytohormones such as gibberellin and cytokinin in flowering plants (Sakamoto et al. 2001, 2006). Class 1 \( KNOX \) genes of \( P. \) patens are
predominantly expressed at the seta meristem of sporophyte but not expressed in gametophyte tissues. Furthermore, cell division in the seta meristem is suppressed in mutant plants that lack class 1 \textit{KNOX}, indicating class 1 \textit{KNOX} genes regulate the seta meristem development during sporophyte development (Sakakibara et al., 2008).

1.2. Hormone involvement in moss development

1.2.1 Auxin

Auxin regulates plant development and physiology. Genetic and molecular biological studies in Arabidopsis have expanded our understanding of the auxin signaling pathway. The auxin signaling pathway is controlled by the regulated degradation of AUX/IAA proteins (Dharmasiri and Estelle, 2004). There are at least 29 AUX/IAA genes in \textit{A. thaliana} genome (Liscum and Reed, 2002; Dharmasiri and Estelle, 2004). The AUX/IAA transcription factors do not interact directly with DNA, but they regulate auxin-mediated gene expression through the interaction with another set of proteins; auxin response factors (ARFs). The interaction between IAA and ARF proteins inactivates the ARF-regulated gene expression of target genes. ARFs contain N-terminal for DNA binding and C-terminal domain that interacts with AUX/IAA factors (Guilfoyle and Hagen, 2001). The N-terminal DNA binding domain of ARF proteins binds to conserved \textit{cis}-element called auxin response elements in genes these proteins regulate. There are 23 ARF genes in the \textit{A. thaliana} genome and the C-terminal domain of most ARFs is conserved except for ARF3 and ARF17 (Liscum and Reed, 2002). ARFs are able to regulate auxin response as activators or repressors. The ARFs which function as transcriptional activator have rich glutamine in the middle region, while ARFs which function as transcriptional repressor contain proline-serine/threonine - rich middle region (Quint and Gray, 2006). AUX/IAA regulates the activities of ARFs by interaction with the ARFs (Tiwari et al., 2003).
Most AUX/IAA proteins turnover rate rapidly (10~80 min) and the short half-lives of AUX/IAA proteins can be extended by MG132 treatment, a proteasome inhibitor while decreased by auxin-treated conditions (Ramos et al., 2001). This indicates that proteasome pathway is involved in the auxin signaling. The SCF\textsuperscript{TIR1} complex positively controls the auxin response by activating ubiquitin-mediated AUX/IAA proteolysis (Gray et al., 2001). SCF\textsuperscript{TIR1} complex is composed of four subunits, ASK1, CUL1, RBX1 and TIR1 (Quint and Gray, 2006). The SCF\textsuperscript{TIR1} complex functions as an E3 ligase for ubiquitin-mediated AUX/IAA proteolysis. It was reported that the conserved 13 amino acids of AUX/IAA domain II is essential for AUX/IAA proteolysis by SCF\textsuperscript{TIR1} complex and the change of the 13 amino acids affected the stability of AUX/IAA proteins (Gray et al., 2001). It is likely that the regulatory mechanism of the auxin signaling pathway has been conserved during land plant evolution; the moss AUX/IAA proteins interact with AtTIR1 protein and silencing of the \textit{PpAFB}, a homologue of Arabidopsis TIR1 induces auxin-resistant mutant phenotypes (Prigge et al., 2010). These results suggest that the molecular mechanism of auxin signaling pathway has been conserved since \textit{P. patens} and \textit{A. thaliana} last shared a common ancestor.

Auxin is involved in moss development; exogenous auxin treatment triggers physiological responses such as the activation of caulonema differentiation and the increase of rhizoid formation (Johri and Desai, 1973; Ashton et al., 1979; Sakakibara et al., 2003), indicating auxin positively regulates caulonema and rhizoid development. Although auxin signal pathway in mosses has not been well studied compared to Arabidopsis, computational approaches using the recently completed genome sequences of \textit{P. patens} suggest that genes required for auxin biosynthesis, auxin signal perception / transduction in seed plants
exists in *P. patens* (Rensing et al., 2008). This hypothesis was supported by some studies with *PpSH1* (homologue of the Arabidopsis SHI/STY family proteins). Arabidopsis SHI/STY family proteins positively regulate auxin biosynthesis (Chandler, 2009; Eklund et al., 2010a). Experimental overexpression of *PpSH1* enhanced auxin concentration and induced auxin response in auxin-untreated condition (Eklund et al., 2010b). This result suggests that the molecular mechanism of auxin biosynthesis has been conserved between *P. patens* and *A. thaliana* during land plant evolution.

The involvement of auxin in caulonema differentiation was also proven by measurement of endogenous auxin levels (Sood and Hackenberg, 1979; Jayaswal and Johri, 1985; Atzorn et al., 1990a, b). Endogenous auxin levels increase as caulonema cells differentiate in the wild type protonema. Furthermore, auxin concentration in mutant plants which show defects in caulonema differentiation is relatively low, compared with the same developmental stage of the wild type. By contrast, auxin concentration in mutant plants (*pg-1*) which show extensive development of caulonema cells is relatively high compared with the same developmental stage of the wild type (Jayaswal and Johri, 1985). Regulation of caulonema cell differentiation by auxin is affected by cyclic AMP (cAMP); auxin-inducing caulonema differentiation was inhibited by exogenous cAMP treatment. Furthermore, *pg-1* mutants which contain high levels of endogenous auxin and extensive development of caulonema cells induce chloronema cells by cAMP treatment. Actually, the concentration of endogenous cAMP in chloronema cells is around five-fold higher than in caulonema cells (Handa and Johri, 1976, 1977). These results show that cAMP is involved in the auxin-mediating developmental transition from chloronema into caulonema cells.
Auxin also positively regulates rhizoid development in the gametophore. Exogenous auxin treatment increases both basal rhizoid and mid-stem rhizoid development; more rhizoids develop in auxin-treated plants than in untreated controls (Ashton et al., 1979; Sakakibara et al., 2003). The $PpHB7$ gene is involved in the auxin-induced rhizoid development. $PpHB7$ encodes a homeodomain-leucine zipper I (HD-Zip I) subfamily protein and regulates rhizoid pigmentation late in rhizoid differentiation; $PpHB7$ is predominantly expressed in rhizoids and mutant rhizoids that lack $PpHB7$ function are pigmentless whereas wild-type rhizoids normally accumulate the pigment (Sakakibara et al., 2003). Furthermore, the transcriptional expression level of $PpHB7$ is regulated by auxin. This suggests that auxin positively regulates rhizoid differentiation by regulating genes required for rhizoid development.

The morphology of caulonema that develop on protonema and rhizoids that develop on gametophores is very similar. A major trait that distinguishes rhizoids from caulonema is pigmentation; mature rhizoids accumulate a brown pigment whereas caulonema are pigmentless. Auxin positively regulates both caulonema and rhizoid differentiation, suggesting it is likely that auxin regulates caulonema and rhizoid differentiation by regulating the expression of genes required both for caulonema and rhizoid differentiation. Two transcription factors ($PpRSL1$ and $PpRSL2$) have been identified that control both differentiation of caulonema and rhizoids in $P. patens$ (Menand et al., 2007b). These genes play key roles in caulonema and rhizoid development; mutant plants which lack $PpRSL1$ and $PpRSL2$ show strong suppression of caulonema and rhizoid differentiation. Nevertheless the regulatory interaction between class 1 RSL genes and auxin in caulonema and rhizoid differentiation is still unknown.
1.2.2 Cytokinin

Cytokinin regulates the developmental transition from protonema to gametophores by controlling the formation of side branch initials and buds. It is hypothesized that developmental regulation by cytokinin is concentration-dependent because picomolar concentrations of cytokinin are enough to activate formation of side branch initial cells and micromolar cytokinin is required for bud formation from the side branch initial cells (Ashton et al., 1979; Bopp and Jacob, 1986). Cytokinin treatment of moss protonema induces the initial cell swelling and chloroplast decrease in size within 2 or 3 hours of cytokinin treatment (Schumakerl and Dietrich, 1997). Recently, a putative cytokinin receptor (PpHK4) which carries a His-Kinase motif was cloned; activity of the PpHK4 progressively increases with the gradual increase of cytokinin concentration treated, indicating this gene is involved in sensing cytokinin in mosses (Ishida et al., 2010). However, it is still unclear because genetic approaches using mutant and overexpressing plants were not tested.

Cytokinin regulates the formation of side branch initial cells and buds in a calcium-dependent manner. Calcium as an intracellular messenger plays a key role in formation of side branch initial and buds in moss development. Calcium positively regulates the developmental process; calcium-free conditions and inhibition of calcium influx by lanthanum suppress the development (Saunders and Helper, 1983). The interaction between cytokinin and calcium in the formation of side branch initials and buds was suggested by investigating membrane-associated calcium concentration using the lipophilic fluorescent calcium chelating probe, chlorotetracycline; the fluorescence was strongly detected at putative side branch forming sites of caulonema cells soon after cytokinin treatment (Saunders and Helpers, 1981). Another experiment using
dihydropyridine (DHP) agonist and antagonist also showed the cytokinin effect on bud formation via voltage-dependent calcium channel (Conrad and Helpers, 1988). DHP are compounds that alter calcium movement through voltage-sensitive channels. One of the DHP stereo-isomers, ((+)202-791), acts as a calcium agonist and another, ((-)202-791), acts as a calcium antagonist. The agonist treatment activates bud formation in the absence of cytokinin whereas the antagonist treatment suppresses cytokinin-inducing bud formation in protonemal filaments. These results indicate that calcium action is required for cytokinin-mediated side branch initial and bud formation.

1.3 Root hair development in Arabidopsis thaliana

Tip-growing caulonema, rhizoids and root hairs form at the interface of the plant and its substratum and function in nutrient acquisition (Peterson and Farquhar, 1996; Gahoonia et al., 1997; Duckett et al., 1998; Gahoonia and Nielsen, 1998). Not only the function in plant growth, but also hormonal and environmental responses are similar between these tip-growing cells; auxin and nutrient-poor conditions activate development of these tip-growing cells. This suggests that molecular and genetic mechanisms controlling these tip-growing cells have been conserved during land plant evolution. This hypothesis was supported by finding that RSL (ROOT HAIR DEFECTIVE SIX-LIKE) gene function is required for the development of these tip-growing cells both in P. patens and A. thaliana (Menand et al., 2007b). Furthermore, root-hairless phenotypes of arabidopsis rsl mutants (Atrhd6 Atrsl1) are rescued by introducing a moss RSL gene (Menand et al., 2007b), suggesting that RSL gene function is conserved between P. patens and A. thaliana, and general molecular and genetic mechanisms might be shared in these tip-growing cell development. Therefore, understanding root hair development which has been well studied
in *A. thaliana* would be important to understand the caulonema and rhizoid development of *P. patens*.

Root hairs are tip growing tubular outgrowths of trichoblasts (Dolan et al., 1994). Root hair development in *A. thaliana* can be understood as having three distinct developmental steps, determination of cell identity, root hair initiation and elongation by tip growth. The determination of root epidermal cell fate is controlled by a transcriptional network including *GLABRA3* (*GL3*), *Enhancer of GLABRA3* (*EGL3*), *TRANSPARENT TESTA GLABROUS* (*TTG*), *GLABRA2* (*GL2*) and *CAPRICE* (*CPC*) (Galway et al., 1994; Rerie et al., 1994; Cristina et al., 1996; Wada et al., 1997; Schellmann et al., 2002; Bernhardt et al., 2003; Bernhardt et al., 2005). *gl3, gl2* and *ttg* mutant plants develop ectopic root hair formation in non hair-forming cell files (N cell files) whereas *cpc* mutant plants decrease the number of root hair formations even in hair-forming cell files (H cell files), indicating that these gene activities regulate the determination of root epidermal cell fate. The expression of these transcription factors is position-dependent in root epidermal cells. This position-dependent hair cell specification results in a striped pattern of hair cell and non-hair cell files along the root axis (Cho et al., 2002).

After the determination of epidermal cell specification trichoblasts start to differentiate root hair formation. Root hair initiation is controlled by another set of genes. Two basic helix loop helix transcript factors, *AtRHD6* and *AtRSL1*, were cloned related to root hair initiation (Masucci and Schiefelbein, 1994; Menand et al., 2007b). The *AtRHD6* and *AtRSL1* genes positively control root hair initiation after the determination of epidermal cell identity. *Atrhd6* single mutant plants exhibit some defects in root hair initiation from trichoblasts along the root axis, whereas *Atrsl1* single mutant plants do not show defects in
root hair development compared to the wild type (Menand et al., 2007b). Expression levels of *AtRHD6* and *AtRSL1* mRNA in the wild type were almost identical to the expression levels of *AtRHD6* and *AtRSL1* mRNA in *Atrsl1* and *Atrhd6* single mutants respectively. This indicates that *AtRHD6* does not regulate *AtRSL1* expression, and *AtRSL1* does not regulate *AtRHD6* expression. The function of *AtRHD6* and *AtRSL1* gene in root hair initiation was clearly proved by analysing the *Atrhd6 Atrsl1* double mutant phenotype and its spatial expression patterns in roots. Mutant plants which lack both *AtRHD6* and *AtRSL1* (*Atrhd6 Atrsl1*) do not develop root hairs in normal growth conditions; phenotypical analysis using a scanning electron microscope (SEM) showed the *Atrhd6 Atrsl1* double mutant does not develop even small bulges along its root axis (Yi et al., 2010). The expression pattern of class 1 *AtRHD6* and *AtRSL1* is position-dependent. *AtRHD6* and *AtRSL1* gene are strongly expressed in H cells but not in N cells (Menand et al., 2007b). The position-dependent expression pattern of these genes is caused by position-dependent regulation by an upstream regulator related to cell fate determination, such as *GL2, GL3* and *CPC*. Mutant plants which lack *GL2* or *GL3* function induce *AtRHD6* expression not only in H cells but also in N cells, whereas mutant plants which lack *CPC* decrease *AtRHD6* expression. These results indicate that class 1 RSL regulates root hair initiation after the determination of root hair cell identity (Lee and Schiefelbein, 2002; Menand et al., 2007b). *AtRHD6* gene as a key regulator in root hair initiation controls other gene expressions which are also involved in root hair initiation such as *AtEXP17* and *AtEXP18* encoding expansin (Cho and Cosgrove, 2002). The expression of these expansin genes is positively regulated by *AtRHD6* genes; *Atrhd6* mutants display the decreased expression level of these expansin genes. This data suggests that *AtRHD6* gene plays key roles in the root hair initiation step in *A. thaliana*. Interestingly, *AtRHD6* regulate root hair initiation independently of auxin which also regulates root hair initiation (Yi et al., 2010).
that \textit{AtEXP17} and \textit{AtEXP18} gene expressions are positively regulated by auxin and \textit{AtRHD6} suggests that separated auxin and \textit{AtRHD6} action regulates common gene functions required for root hair initiations.

It is likely that \textit{AtRHD6} is also involved in the determination of the root hair forming site in root hair initiation. The mutant plants which lack \textit{Atrhd6} show abnormality in the site of root hair emergence. The root hairs of the wild-type \textit{A. thaliana} develop from the distal end of the epidermal cells (Schiefelbein and Somerville, 1990). In contrast to wild type, \textit{Atrhd6} mutants form root hairs more basally on the hair forming epidermal cells than in the wild type, indicating \textit{AtRHD6} is also involved in the determination of the root hair forming site (Masucci and Schiefelbein, 1994). Arabidopsis \textit{Rop2 GTPase} is also involved in the root hair initiation by determining the root hair-forming sites in trichoblasts; GFP fused Rop2 proteins are highly accumulated in the future site of hair formation before the formation of small bulges (Jones et al., 2002). However, the possible interaction between \textit{AtRHD6} and \textit{Rop2-GTPase} in the determination of root hair forming sites is still unknown.

Root hair initials elongate by tip growth. \textit{AtRSL2} and \textit{AtRSL4} genes are were cloned related to the root hair elongation. \textit{AtRSL2} and \textit{AtRSL4} encode basic helix loop helix transcription factors which function in controlling root hair elongation after root hair initiation; mutant plants that lack \textit{AtRSL2} and \textit{AtRSL4} suppress elongation of root hairs and develop only small bulges, which do not elongate and develop into mature root hairs (Yi et al., 2010). Furthermore, \textit{AtRSL4}-overexpressing transgenic plants (\texttt{35S:AtRSL4}) develop very long root hairs compared to wild type root hairs, suggesting that \textit{AtRSL4} regulates root hair elongation (Yi et al., 2010). \textit{AtRSL2} and \textit{AtRSL4} genes are expressed in trichoblasts after the trichoblasts develop root hairs. Transcriptional expressions of these
genes are positively regulated by \textit{AtRHD6} and \textit{AtRSL1} which control root hair initiation. These results suggest that regulation of \textit{AtRSL2} and \textit{AtRSL4} by \textit{AtRHD6} and \textit{AtRSL1} controls root hair elongation.

Root hair elongation also requires rearrangement of actin cytoskeleton. Involvement of actin dynamics in root hair elongation was supported by actin dynamics-mediating gene studies. ADF (actin depolymerising factor) controls actin dynamics by increasing the rate of actin dissociation (Carlier et al., 1997). Transgenic plants that overexpress \textit{AtADF1} develop relatively short and thick root hairs compared to wild type. Another actin dynamic regulator, \textit{AtPFN1} (profilin 1) is an actin-binding protein (Ramachandran et al. 2000). Experimental overexpression of \textit{PFN1} gene increases root hair length while overexpression of antisense \textit{PFN1} decreases the length. These results suggested actin dynamics is involved in root hair elongation. Visualization of F-actin accumulation at tips of young root hairs and inhibition of root hair elongation by latrunculin B, an actin depolymerisation drug, clearly supported that actin dynamics is required for root hair elongation (Baluska et al., 2000). Transgenic plants expressing GFP-fused mouse talin protein which labels F-actin shows high intensity of GFP signal at tips of young root hairs, and latrunculin B treatment strongly suppresses root hair elongation by activating depolymerisation of actin filaments. Furthermore, profilin is expressed in the small bulges in which F-actin highly accumulates, proving that actin dynamics is important for root hair elongation. Plant cytoskeleton consists of actin microfilaments and microtubules. During root hair development, microtubules function in the nucleus movement and the maintenance of the growth direction but are not required for tip growth of root hairs unlike actin cytoskeleton. The hair-forming bulges are initially depleted in microtubules. Later, nuclear-associated microtubules emerge in the root hair and become involved in the
maintenance of growth direction (Baluska et al., 1997, 1998, 2000); oryzalin and taxol treatment of wild type root induces curvy root hairs whereas growth rate is identical to the control (Bibikova et al. 1999).

Root hair elongation also requires some cations and reactive oxygen species (ROS). Potassium ion (K\(^+\)) is an active cation in root hair development. Potassium ion influx, which is controlled by the plasma membrane proton pump and potassium channel protein, is observed in growing root hairs, which is sufficient to induce cellular expansion (Lew, 1991). Calcium ions (Ca\(^{2+}\)) also play key roles in root hair tip growth. Cytoplasmic free Ca\(^{2+}\) is accumulated at the actively growing root hair tips. Inhibition of Ca\(^{2+}\) transport across the plasma membrane suppresses tip growth of root hairs (Schiefelbein et al., 1992; Wymer et al., 1997). ROS are also involved in root hair elongation. AtRHD2 encoding NADPH oxidase regulates root hair elongation by controlling ROS biosynthesis in root hair development (Foreman et al., 2003). ROS production in root hair cells has an effect on Ca\(^{2+}\) accumulation in root hairs. Mutant plants that lack AtRHD2 form short root hairs without elongation and Ca\(^{2+}\) is not accumulated in the root hairs, indicating that regulatory interaction between Ca\(^{2+}\) and ROS is involved in root hair elongation (Wymer et al., 1997; Foreman et al., 2003).

1.4 Auxin and root hair development

Auxin is one of the most well characterized plant hormones related to root hair development. Auxin positively regulates root hair development; exogenous auxin treatment increases root hair length and auxin-response mutant plants such as axr1, axr2 and axr3 are affected in root hair development (Lincoln et al., 1990; Wilson et al., 1990; Timpte et al., 1995; Leyser et al., 1996; Pitts et al., 1998). Auxin is specifically involved in
root hair initiation (Masucci and Schiefelbein, 1996; Knox et al., 2003; Grebe et al., 2002) and elongation (Pitts et al., 1998; Lee and Cho., 2006).

Auxin regulates root hair development in a concentration-dependent manner. Endogenous auxin levels are regulated by the polar auxin transporting system using auxin influx and efflux carriers; NPA, auxin efflux inhibitor, treatment increases auxin concentration in root hair cells, which results in longer root hairs, compared to an untreated control (Cho et al., 2007a and b). Similarly, the experimental overexpression of PINOID, PIN2 and PGP4 which positively regulate auxin efflux induces suppression of root hair development (Friml, 2003; Wiśniewska et al., 2006; Cho et al., 2007b). By contrast, experimental overexpression of AUX1 which regulates auxin influx increases root hair length and aux1 mutant plants suppress root hair development (Cho et al., 2007b; Jones et al., 2009). Interestingly a recent study about DR5 promoter activity in the A. thaliana roots showed that the concentration of endogenous auxin in atrichoblasts is relatively high compared with trichoblasts; transgenic plants transformed with DR5 promoter:GFP show stronger intensity of GFP in atrichoblasts than trichoblasts in wild type, and the difference in GFP intensity between atrichoblasts and trichoblasts doesn't exist in aux1 mutant (Jones et al., 2009). This suggests that auxin accumulation in atrichoblasts is not sufficient to differentiate root hairs from atrichoblasts, and auxin does not have an effect upon the determination of hair-forming cell identity in A. thaliana.

Auxin effect on root hair initiation was studied in mutant plants which lack AtRHD6 and AtRSL1. Mutant plants which lack both AtRHD6 and AtRSL1 function (Atrhd6 Atrsl1) do not differentiate root hairs along the root axis in normal growth conditions, whereas auxin treatment induces root hair differentiation in Atrhd6 Atrsl1 double mutants. Furthermore
auxin treatment does not affect on the transcriptional expression of \textit{AtRHD6} and \textit{AtRSL1}. These results indicate that auxin regulates root hair initiation independently of \textit{AtRHD6} and \textit{AtRSL1}. Instead, auxin positively regulates a transcriptional expression level of \textit{AtRSL4} which controls root hair elongation by tip growth. In contrast to \textit{Atrhd6 Atrsl1} double mutants, mutant plants which lack both \textit{AtRSL2} and \textit{AtRSL4} are resistant to auxin; auxin treatment does not induce root hair elongation in \textit{Atrsl2 Atrsl4} double mutants (Yi et al., 2010) indicating that auxin regulates root hair elongation dependently on \textit{AtRSL4}. This is consistent with previous studies suggesting that auxin controls root hair initiation and elongation downstream of \textit{GL2} and \textit{TTG} (Masucci and Schiefelbein, 1996 Pitts et al., 1998).

1.5 Class 1 \textit{RSL} gene and caulonema and rhizoid development

\textit{ROOT HAIR DEFECTIVE SIX-LIKE (RSL)} genes encode basic helix loop helix transcription factors. There are six \textit{RSL} genes in \textit{A. thaliana} (figure 1.2). \textit{AtRHD6} and \textit{AtRSL1} belong to class 1 \textit{RSL} gene clade, and \textit{AtRSL2}, 3, 4 and 5 belong to class 2 \textit{RSL} gene clade (Menand et al., 2007b). In \textit{A. thaliana}, \textit{RSL} gene regulation and function have been well understood related to root hair development; class 1 \textit{RSL} regulates root hair initiation independently of auxin while class 2 \textit{RSL} controls root hair elongation dependently of auxin. Similarly, \textit{P. patens} has seven \textit{RSL} genes. \textit{PpRSL1} and \textit{PpRSL2} belong to class 1 \textit{RSL} gene clade, and five \textit{PpRSL} genes (\textit{PpRSL 3, 4, 5, 6 and 7}) belong to class 2 \textit{RSL} gene clade (Menand et al., 2007b); class 1 \textit{PpRSL} genes are involved in caulonema and rhizoid development whereas class 2 gene function in moss development is still unknown. Despite the clear involvement of class 1 \textit{RSL} in caulonema and rhizoid development, their regulation mechanisms including interaction with auxin is unknown in moss development.
Previous characterization of the class 1 RSL genes indicates that their function has been conserved between P. patens and A. thaliana during land plant evolution since land plants last shared a common ancestor 416 million years ago. P. patens and A. thaliana mutants that lack class 1 RSL gene function fail to develop rhizoids and root hairs respectively. Despite the demonstration that the function of these genes is conserved between A. thaliana and P. patens, the mechanism of their regulation remained unknown. In particular, it is known that auxin regulates rhizoids and caulonema development in moss but the regulatory relationship between auxin and a class 1 RSL gene is unknown. Furthermore after plants established the first continental vegetation 470 million years ago, there was explosive increase of morphological diversity by gene modification and rearrangement of gene regulatory systems (Kenrick and Crane, 1997; Bateman et al., 1998; Davison and Erwin; 2006; Richardt et al., 2007; Peter and Davison; 2011). This suggests the possibility that the RSL regulation mechanism changed during land plant evolution despite the conserved function. The aim of the research reported in this thesis is to test the hypothesis. Specifically I set out to understand the regulatory relationship between auxin and class 1 RSL gene in P. patens and compare it with the mechanism that exists in A. thaliana.
Figure 1.2 A phylogenetic tree showing the relationships between *A. thaliana* and *P. patens* RSL proteins.
Chapter Two:

Materials and Methods
2.1 Plant materials and growth

The Gransden wild type strain of *P. patens* (Hedw) Bruch and Schimp, was used in this study (Ashton et al., 1979). Cultures were grown at 25°C and illuminated with a light regime of 16/8 h (light/dark) and a quantum irradiance of 40 μmol m^{-2} s^{-1}. For the growth of gametophores, spores were inoculated on a 9 cm Petri dish containing solid minimal media overlaid with a cellophane disk (AA packaging) for the indicated time. Solid minimal media includes 0.8 g/l Ca(NO₃)₂4H₂O, 0.25 g/l MgSO₄7H₂O, 0.0125 g/l FeSO₄7H₂O, 0.055 mg/l CuSO₄5H₂O, 0.055 mg/l ZnSO₄7H₂O, 0.614 mg/l H₃BO₃, 0.389 mg/l MnCl₂4H₂O, 0.055 mg/l CoCl₂6H₂O, 0.028 mg/l KI, 0.025 mg/l Ne₂MoO₄2H₂O and 25 mg/l KH₂PO₄, and were titrated to pH7 with 4M KOH. Protonemal samples for transformation were grown on minimal media supplemented with 5 mg/l NH₄ and 50 mg/l glucose for 1 week. *P. patens Pprs11, Pprs12* and *Pprs11 Pprs12* double mutants, as well as *A. thaliana Atrhd6 Atrsl1* lines were previously described (Menand et al., 2007b).

2.2 Phenotypical analysis and measurement

For the phenotypic analysis of caulonema development, spores for *Pprs11* single, *Pprs12* single and *Pprs11 Pprs12* double mutant and protoplasts for 35S:PpRSL1;35S:PpRSL2 were placed on solid minimal media overlaid with cellophane disks. For the phenotypic analysis of rhizoid development, small protonemal inocula were placed on solid minimal media overlaid with cellophane disks and grown for 3 weeks. Protonema and gametophore images were captured with a Nikon Coolpix 995 camera mounted on a Leica Wild M10 stereomicroscope and with a Pixera Pro ES600 camera mounted on a Nikon Eclipse 800 and measured using Photoshop 7.0(Adobe). Cell cycle times were determined by calculating rates of cell production. Cell production rates were calculated by dividing the
time interval (in hours) during which colonies were growing by the number of cells produced in protonemal filaments during that time interval. Cell numbers were determined by counting the numbers of cross walls that formed in a filament.

2.3 Auxin, TIBA and NPA treatment

Auxin treatment of *P. patens* protonema was carried out by plating spores in solid minimal media supplemented with 1 µM α-naphthalene acetic acid and incubating for 2 weeks. For the study of rhizoids, auxin treatment was carried out by transferring 3-week-old plants to solid minimal media supplemented with NAA and incubating for 1 week. TIBA and NPA treatment of protonema was carried out by plating spores in solid minimal media supplemented with 100 µM TIBA and 20 µM NPA for 1 week. For the study of TIBA effect upon rhizoid development, TIBA treatment was carried out by transferring 3-week-old plants to solid minimal media supplemented with 300 µM TIBA and incubating for 1 week.

2.4 Construction of a constitutively overexpressing vector

For constitutive overexpression of both *PpRSL1* and *PpRSL2* at the same time, a 35S:PpRSL1;35S:PpRSL2 vector was generated. To obtain 35S:PpRSL1:NOSTer DNA cassette including *Spe*I and *Apa*I enzymatic restriction sites, two different vectors were used; pCAMBIA-35S:PpRSL1 carrying 35S:PpRSL1:NOSTer cassette (Menand et al., 2007b) and pBluescript II SK+ (Stratagene) having *Spe*I and *Apa*I sites. First, pCAMBIA-35S:PpRSL1 was excised with *Eco*RI and *Hind*III to obtain 35S:PpRSL1:NOSTer cassette and this cassette was inserted into the pBluescript vector which had been digested with

35
EcoRI and HindIII. pBluescript-35S:PpRSL1 carrying SpeI – 35S:PpRSL1:NOS_Ter – Apal cassette was obtained by ligation. The SpeI – 35S:PpRSL1:NOS_Ter – Apal cassette, which was obtained by digesting pBluescript-35S:PpRSL1 with SpeI and Apal, was inserted into pPpRSL1-KO vector carrying partial genomic DNA fragments of PpRSL1 for homologous recombination (Menand et al., 2007b). The vector was named 35S:PpRSL1. To obtain 35S:PpRSL2:NOS_Ter cassette carrying NotI and AapI sites two different vectors, pCAMBIA-35S:PpRSL2 carrying 35S:PpRSL2:NOS_Ter cassette and pGEM-T Easy (Promega) including NotI and Apal sites were used. pCAMBIA-35S:PpRSL2 was obtained by amplifying the PpRSL2 coding sequence from protonema cDNA with 35S:PpRSL2 primers and cloning it into the BamH1 and SalI sites of pCAMBIA 1300 (Menand et al., 2007b). 35S:PpRSL2: NOS_Ter cassette was obtained by digesting pCAMBIA-35S:PpRSL2 with EcoRI and SphI. This cassette was inserted into pGEM-T Easy vector which had been self-ligated and digested with EcoRI and SphI. NotI-35S:PpRSL2:NOS_Ter- Apal cassette from pGEM-35S:PpRSL2 vector was inserted into NotI and Apal sites of 35S:PpRSL1.

2.5 Construction of PpRSL1_promoter:GUS and PpRSL2_promoter:GUS vectors

GUS transcriptional reporter vectors were generated by inserting two genomic DNA fragments of PpRSL1 and PpRSL2 promoter regions into pBHSNR-GUS vector carrying GUS-35S_Ter. pBHSNR-GUS vector was constructed by inserting GUS coding sequence and 35S terminator into ClaI/SpeI sites and BglII/Apal sites of pBHSNR vector (Menand et al., 2007b), respectively. Two genomic DNA fragments of PpRSL1 gene were cloned by PCR. The genomic DNA fragment of PpRSL1 promoter (634 bp) was inserted into AscI and ClaI sites of pBHSNR-GUS and another fragment directly downstream of PpRSL1
promoter region was inserted into BamHI and HindIII sites of pBHSNR-GUS. For the PpRSL2 promoter: GUS vector construction two genomic DNA fragments of PpRSL2 gene were amplified by PCR. The genomic DNA fragment of PpRSL2 promoter (905 bp) was inserted into AscI and ClaI sites of pBHSNR-GUS and another fragment directly downstream of PpRSL2 promoter region was inserted into SmaI and HindIII sites.

2.6 Physcomitrella patens transformation

To generate the transgenic moss plants including 35S:PpRSL1;35S:PpRSL2, PpRSL1 promoter:GUS and PpRSL2 promoter:GUS, gene targeting system based on homologous recombination were used. In homologous recombination, two homologous DNA strands exchange their genetic materials. The gene targeting system based on homologous recombination makes it possible to modify a specific area of genome the without changing or damaging the genome (figure 2.1). The DNA constructs for homologous recombination contain two extra DNA fragments used for recognition of the modification sites. The recombination efficiency improves with the increase of length of the homologous DNA fragments (Kamisugi et al., 2005). It is also thought that linearization of DNA construct also improve the transformation efficiency. Linearized DNA constructs are introduced into moss protoplasts by PEG-mediated transformation technique as described previously by Schasfer and Zrýd (1997). Four plates of protonemal tissues grown on nutrient rich media for 1 week were collected. 25 ml driselase was treated and incubated for 30-40 minutes with gentle mixing. For collecting protoplasts, samples were filtered with microspore sieve and 8.5% mannitol. Collected protoplasts were suspended in 6 ml MMM solution (8.5% Mannitol, 15ml Magnesium chloride and pH 5.6 of 0.1% MES). 300 µl of protoplasts were added into a 50 ml tube which already had the DNA constructs, and mixed gently. 300 µl
Figure 2. 1. Schematics showing gene targeting based on homologous recombination

(Prigge et al., 2010)
of PEG was added to the DNA and protoplasts and mixed. After conducting a heat shock at 45°C for 5 min, the protoplasts were left at room temperature for 10 min and 6.5 ml of liquid nutrient rich media was added including 8.5% mannitol. After overnight incubation under dark conditions, the protoplasts were placed onto solid nutrient rich proto media with the aid of top agar (1.4 % agar and 8.5% mannitol). The 35S:PpRSL1;35S:PpRSL2 vector and 35S:PpRSL1 vector were linearised with AvrII and SwaI before protoplast transformation whereas 35S:PpRSL2 vector was linearised with SwaI. Transformants for 35S:PpRSL1;35S:PpRSL2 and 35S:PpRSL1 were selected on G418 (50 μl/ml) and transformants for 35S:PpRSL2 were selected on Hygromycin B (25 μl/ml). For the transformations of PpRSL1 promoter: GUS and PpRSL2 promoter: GUS, PpRSL1 promoter: GUS vector was linearised with AscI and HindIII before protoplast transformation whereas the PpRSL2 promoter: GUS vector was linearised with AscI and BamHI. Transformants were selected on Hygromycin B (25 μl/ml).

2.7 RT-PCR analysis

The total RNA from 2–week-old protonema and 4-week-old gametophores was extracted with the RNeasy Plant Mini Kit (Qiagen). 1 μg of total RNA was used for cDNA synthesis with the Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primers. PCR was carried out with equivalent amounts of cDNA template for amplification of fragments of PpRSL1 and PpRSL2 and GAPDH (X72381).

2.8 GUS staining analysis, embedding and sectioning

Isolated gametophores were incubated in GUS staining solution (100 mM NaPO₄ [pH 7.0], 1mM 5-bromo-4-chloro-3-indolyl-glucuronide, 1 mM potassium ferricyanide and 0.2% Triton X-100) at 37°C for 2 – 24 hours. The samples were then washed with 100 mM
NaPO₄ [pH7.0] and incubated in 70% EtOH. Whole mounted samples were imaged with a Nikon Coolpix 995 camera mounted on a Leica Wild M10 stereomicroscope. For transverse sectioning, gametophores were aligned on a 1 mm layer of solid 1% agarose and covered with 1% molten agarose. After solidification, samples were cut into small blocks and washed twice in double distilled H₂O for 15 minutes, then dehydrated in a graded series of ethanol (25, 50, 75 and 100%), each for 30 minutes. The dehydrated samples were sequentially incubated in a series of Technovit 7100 cold-polymerizing resin (33, 66 and 100% (v/v) in EtOH), each for 1 hour. Samples were then incubated in 100% Technovit for 1 day and placed in plastic moulds. To solidify samples a mixture of Technovit and hardener solution II (v/v=15:1) was treated at room temperature for 1 day. 10 µm sections were taken from gametophores with an Ultracut E (Reichert-Jung). Images were captured with a Pixera Pro ES600 camera mounted on Nikon Eclipse 800.

### 2.9 Genomic DNA extraction from *Physcomitrella patens* for PCR

Genomic DNA extraction was carried out as described previously by Roger and Bendich (1988), with slight modification. 1 gram of protonemal tissue was grinded in liquid nitrogen. 1 ml of CTAB solution (2% CTAB, 100mM Tris [pH8.0], 20mM EDTA [pH8.0], 1.4M NaCl and 1% PVP) which was pre-heated at 65°C was added and incubated at 65°C for 15 min. 1 volume of CHCl₃/isoamyl (24:1) alcohol without phenol was added to the samples. After centrifugation, supernatant was transferred into new tubes and 10 % CTAB (10 % CTAB and 0.7M NaCl) was added. After purification with 1 volume of CHCl₃/isoamyl, collected supernatant was added with 1 volume of CTAB precipitation solution (1% CTAB, 50mM Tris [pH8.0], 10mM EDTA [pH8.0]) and incubated at room temperature for 1 hour. Pellets collected by centrifugation were resolved in HSTE solution.
(10mM Tris [pH8.0], 1mM EDTA [pH8.0] and 1M NaCl) at 50°C. Final genomic DNA was collected by ethanol precipitation.

2.10 Southern blot analysis

The total genomic DNA from protonemal tissues was isolated using the Nucleon Phytopure DNA extraction kit (Amersham Biosciences). 8 µg of DNA was digested with HindIII and run on a 0.7% agarose gel and transferred to a nitrocellulose membrane in alkaline condition (0.4N NaOH and 1M NaCl) at room temperature for 14 hours. $^{35}$P-labeled NPT was prepared by random primer extension with $[\alpha-^{32}\text{P}]$ dATP. For the hybridization church buffer was used (0.5 M Na-Phosphate, 0.1 mM EDTA, 7% SDS and 1% BSA ). Hybridization was carried out at 65°C for 24 hours and washed four times with washing solution. The X – films were exposed for 1 and 7 days.
Chapter Three:

Class 1 \textit{RSL} function

in caulonema differentiation

of \textit{Physcomitrella patens}
3.1 Summary

Protonema that develops from haploid spores is the filamentous stage of *P. patens*. Protonema comprises two different cell types - chloronema and caulonema. Both class 1 *RSL* genes and auxin regulate the caulonema differentiation, but the regulatory relationship between class 1 *RSL* and auxin is unknown in moss protonema development. This study shows that chloronema cells in proximal regions are becoming progressively more caulonemal distally with each round of apical cell division. The gradual transition from chloronema to caulonema causes gradient cell growth along filaments. Class 1 *RSL* genes regulate the gradient cell growth by regulating the developmental transition from chloronema to caulonema; mutant plants which lack class 1 *RSL* functions (*Pprsl1Pprsl2*) do not develop gradient cell identity along protonemal filaments. The transcriptional expressions of class 1 *RSL* genes are positively regulated by auxin. Furthermore, mutant plants which lack both *PpRSL1* and *PpRSL2* function do not differentiate caulonema even in auxin-treated conditions, whereas constitutive co-expression of *PpRSL1* and *PpRSL2* is sufficient to activate constitutive caulonema development. Together these data suggest that auxin regulates caulonema differentiation by positively regulating *PpRSL1* and *PpRSL2*. 
3.2 Introduction

Two generations alternate during the life cycle of *P. patens*. The gametophyte generation is the haploid phase of the life cycle, and the sporophyte generation is a diploid phase. Protonema development is the first phase in the haploid stage of the life cycle after spore germination. The protonema is the multicellular filamentous stage of the gametophyte. Protonemal filaments comprise two different types of cells; chloronema and caulonema. Chloronema having numerous chloroplasts and perpendicular cell walls are produced by apical cell division at the tips of protonemal filaments with a cell cycle time of approximately 24 hours (Cove and Knight, 1993; Schween et al., 2003; Cove, 2005). At some point, chloronema cells develop into caulonema cells at the growing edge of protonema. Caulonema have few chloroplasts that are smaller than those of chloronema and their cross walls are oblique instead of transverse. New cells are produced by division of apical cells with a relatively short cell cycle time of approximately 7 hours (Cove and Knight, 1993; Schween et al., 2003; Cove, 2005).

Differentiation is the process by which specialized cell types form during development of multi-cellular organisms. Diverse cell types develop in various positions in the body of an organism, because of the spatially-controlled expression and activity of regulators such as hormones and transcription factors. The differentiation of caulonema from chloronema is positively regulated by auxin and class 1 *RSL* genes; auxin treatment of wild type protonema activates caulonema differentiation from chloronema, and the differentiation of caulonema from chloronema is defective in mutants which lack the function of class 1 *RSL* (Johri and Desai, 1973; Ashton et al., 1979; Menand et al., 2007b). Despite crucial roles of auxin and class 1 *RSL* genes in caulonema differentiation, the regulatory interaction between auxin and class 1 *RSL* genes have been poorly understood in *P. patens*. In this
study, my aim is to understand the regulatory relationship between auxin and class 1 \textit{RSL} in caulonema development.

To understand the regulatory relationship between auxin and class 1 \textit{RSL} genes is also very important. Although a previous study showed that class 1 \textit{RSL} function in rooting cell development is conserved between \textit{P. patens} and \textit{A. thaliana}, it is still unknown if the mechanism of class 1 \textit{RSL} regulation was conserved during the land plant evolution. It is known that the modification of gene regulatory networks gave rise to the morphological diversity and complexity during evolution (Davison and Erwin; 2006; Peter and Davison; 2011). This suggests that the regulatory interaction between auxin, a regulator of caulonema development, and class 1 \textit{RSL} genes may be different in \textit{P. patens} and \textit{A. thaliana}. Therefore, to elucidate the regulatory relationship between auxin and class 1 \textit{RSL} genes in \textit{P. patens} is a central aim of this research presented in this thesis.

3.3 Results

3.3.1 There is a gradual transition from chloronema to caulonema in moss protonema

Germinating spores of \textit{P. patens} develop filamentous networks, protonema. Protonemal filaments consist of two distinct cell types, chloronema and caulonema (figure 3.1). Chloronema are slow growing filamentous cells with numerous chloroplasts and perpendicular cell walls, while caulonema are rapid growing filamentous cells with few chloroplasts and oblique cell walls. Both filamentous cells grow at their tips by tip growth and cell division (Reski, 1998). In germination spores produced $2 (2.17 \pm 0.39 \text{ (S.D.) n=}$
Figure 3.1 Gradient cell identities along protonemal filaments.

A. Germinating spore with two emerging filaments.

B. Protonema 7 days after germination.

C. Chloronema cell with a transverse cell wall (arrow).

D. Caulonema apical cell with an oblique cell wall (arrow).

E. Change in cell length and width along a filament; cell 1 is the proximal-most cell that forms upon germination, and the sub-apical cell is the distal-most differentiated cell along a filament.

F. Relationship between cell length and width along the proximal-distal protonemal filament axis. Asterisk indicates significant difference between length of 1st, 5th and sub-apical cells based on 95% confidence intervals.

Scale bars = 50 µm in (a); 100 µm in (b), (c) and (d).
12) protonemal filaments (figure 3.1A). The first cells to differentiate in these filaments were chloronemal; they were short and have transverse cross walls (figure 3.1A, C). As protonemal filaments grow, caulonema started to differentiate from chloronema at the growing edges of colonies. After germination within 1 week, cell number in filaments increased to 9 or 10 (9.6 ± 0.52 (S.D.)). Cells in distal regions were longer and narrower than the chloronema cells in the proximal region. Furthermore, the distal cells in the 1 week old filaments developed oblique cross walls, indicating that there is a transition from chloronema to caulonema within the first week of protonemal development. Interestingly, fifth cells from the proximal region displayed a transitional morphology between chloronema in the proximal regions and caulonema in the distal region of the protonemal filaments; the fifth cell was longer and narrower than the first cells in the proximal region but shorter and thicker than sub-apical cells (figure 3.1E, F). This data supports the hypothesis that chloronema gradually develop into caulonema with each increase of each round of apical cell division.

To determine how this transition occurs in protonemal filament development, the change in cell length was characterised along a 7 day-old protonemal filament, because the developmental transition from short chloronema into long caulonema should be accompanied by an increase in cell length along the filament. Cell length gradually increased along the filament (figure 3.3M). This supports that caulonema gradually differentiate from chloronema in protonemal filament development.

The gradual transition from chloronema into caulonema was independently tested by characterizing the cell cycle times of cell in protonemal development. Chloronema and caulonema are present for different durations in the cell cycle; slow-growing chloronema
cells approximately divide every 24 hours whereas rapid-growing caulonema cells divide every 7 hours (Cove and Knight, 1993; Schween et al., 2003). If the developmental transition from chloronema to caulonema occurs gradually, it would be expected that the cell cycle times would gradually decrease with the increase of each round of apical cell division. The cell cycle of the first three cells which developed between germination and the 3 day of filamentation was approximately 24 hours. Cell cycle times gradually decreased in apical cells which formed later in protonemal development; from 18.9 hours at 4 days to 12.1 hours at 10 days (table 3.1). This data clearly indicates that chloronema cells gradually develop into caulonema in the protonemal development of *P. patens*.

### 3.3.2 *PpRSL1* and *PpRSL2* are required for caulonema differentiation from chloronema in protonema development

The developmental transition from chloronema to caulonema requires some gene expression. For example, the *PpARPC1* encoding subunit of the actin-related protein2/3 (Arp2/3) complex and the *PpHXK1* encoding hexokinase, are required for caulonema differentiation; each mutant plant which lack *PpARPC1* or *PpHXK1* suppress rapidly elongating caulonema differentiation at the growing edge of protonemal filaments (Harries et al., 2005; Thelander et al., 2005). However, both *Pparpc1* and *Pphxk1* mutant protonema are also affected in chloronema development. This suggests that these genes are not specific regulators controlling caulonema differentiation from chloronema.

*PpRSL1* and *PpRSL2* regulate caulonema differentiation; mutant plants which lack both *PpRSL1* and *PpRSL2* strongly inhibit caulonema differentiation from chloronema in protonemal development (Menand et al., 2007b). However, it is unknown if chloronema
Table 3.1 Cell cycle times become shorter during the chloronema-to-caulonema transition.

<table>
<thead>
<tr>
<th></th>
<th>0-3day</th>
<th>3-4 day</th>
<th>3-6 day</th>
<th>3-8 day</th>
<th>3-10 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>24.4 ± 4.60 hr</td>
<td>18.9 ± 6.41 hr</td>
<td>15.9 ± 1.95 hr</td>
<td>13.1 ± 1.56 hr</td>
<td>12.1 ± 0.6 hr</td>
</tr>
<tr>
<td>prs1pr2</td>
<td>24.0 ± 3.79 hr</td>
<td>24.0 ± 0 hr</td>
<td>24.8 ± 5.01 hr</td>
<td>25.1 ± 3.63 hr</td>
<td>24.4 ± 3.25 hr</td>
</tr>
</tbody>
</table>
development also requires class 1 RSL gene functions or not. If class 1 RSL is not involved in chloronema development but specifically regulates caulonema differentiation from chloronema, mutant plants which lack \( PpRSL1 \) and \( PpRSL2 \) would develop morphologically identical chloronema cells to those of the wild type. To determine that class 1 RSL genes specifically control caulonema differentiation, a phenotypical analysis was carried out with 7 day old wild type plants, \( Pprsl1 \), \( Pprsl2 \) single and \( Pprsl1 Pprsl2 \) double mutants. Morphologies of \( Pprsl1 \) and \( Pprsl2 \) single mutants were almost identical to wild type (figure 3.2A, B, C, E); \( Pprsl1 \) and \( Pprsl2 \) single mutants differentiated caulonema cells at the distal region of protonemal filaments. Cell lengths of the first chloronema cells and sub-apical caulonema cells between wild type and each \( Pprsl1 \) and \( Pprsl2 \) single mutants were almost same. However, \( Pprsl1 Pprsl2 \) double mutants strongly suppressed caulonema differentiation in the distal region and all cells which developed on protonemal filaments of \( Pprsl1 Pprsl2 \) double mutants were chloronemal (figure 3.2D, E); cell lengths between first proximal cells and sub-apical cells were almost identical in \( Pprsl1 Pprsl2 \) double mutants unlike \( Pprsl1 \), \( Pprsl2 \) single mutants and wild type. Furthermore, the first chloronema cell length of \( Pprsl1 Pprsl2 \) double mutants was almost the same to the first chloronema cell length of wild type, indicating that chloronema development does not require the class 1 RSL gene function. This data also suggests that class 1 RSL specifically regulates the developmental transition from chloronema to caulonema in protonema development.

### 3.3.3 Class 1 RSL controls gradient cell growth along filaments by regulating caulonema differentiation
Figure 3. 2 All cells in the protonemal filaments of *Pprsl1* *Pprsl2* double mutants are chloronema.

A. Protonema of 1 week old of wild type.
B. Protonema of 1 week old *Pprsl1* single mutants.
C. Protonema of 1 week old *Pprsl2* single mutants.
D. Protonema of 1 week old *Pprsl1* *Pprsl2* double mutants.
E. First and sub-apical cell lengths in WT, *Pprsl1*, *Pprsl2* single and *Pprsl1* *Pprsl2* double mutants. Asterisk indicates significant difference between length of 1st and sub-apical cells based on 95% confidence intervals.

Scale bars = 100 μm.
Because class 1 RSL function is required for the developmental transition from chloronema to caulonema in protonema development, it was expected that there would be no morphological differences between young protonema of the wild type and Pprsl1 Pprsl2 double mutants, because the early developmental stage of protonemal filaments mainly develops chloronema cells (Cove and Knight, 1993). To test this hypothesis a phenotypical analysis was carried out using different developmental stages of the wild-type protonema and Pprsl1 Pprsl2 double mutants. As expected the 3 day old protonema of Pprsl1 Pprsl2 double mutants were almost identical to the wild-type protonema in morphology. (figure 3.3A, G). All cells which developed between germination and 3 days in the wild-type filaments and Pprsl1 Pprsl2 mutant filaments were chloronema; all cells which develop both in wild type and Pprsl1 Pprsl2 mutant filaments were short and had perpendicular cell walls. By 4 days after germination wild type filaments started to differentiate caulonema-like cells in the distal regions, whereas Pprsl1 Pprsl2 double mutants developed only chloronema cells along the filaments. With the increase of each round of caulonema cell division, the difference in size between protonemal colonies of wild type and Pprsl1 Pprsl2 double mutants gradually increase, because Pprsl1 Pprsl2 double mutants suppress caulonema cells along the filaments and develop only chloronema cells which grow and divide with a relatively low growth rate and long cell cycle time. By 18 days after germination, the protonema of the wild type was around two times bigger in size than the protonema of the Pprsl1 Pprsl2 mutants. (figure 3.3F, L).

If caulonema differentiation in the Pprsl1 Pprsl2 protonemal filaments is completely suppressed it would be expected that Pprsl1 Pprsl2 double mutants would not develop the gradient filamentous cell growth which develops in the wild type. To test this hypothesis, the change in cell length was characterised along a 7 day-old protonemal filament of the
Figure 3. 3 *Pprsl1Pprsl2* double mutants do not develop gradient cell growth along filaments

**A-F.** Wild-type protonemal morphology at different stages of development.

**G-L.** *Pprsl1 Pprsl2* double mutant protonema at different stages of development.

**M.** Relationship between cell length and position along proximal-distal axis in wild type and *Pprsl1Pprsl2* double mutant protonemal filaments.

**N.** Increase in cell numbers of wild type and *Pprsl1 Pprsl2* double mutant with age (0 – 10 days). Asterisk indicates significant difference between wt and *Pprsl1Pprsl2* based on 95 % confidence intervals.

Scale bars = 100 µm.
Ppsl1 Ppsl2 double mutant. As expected, the Ppsl1 Ppsl2 double mutant did not develop gradient cell growth along protonemal filaments (figure 3.3M); all cells which develop between the first proximal cells and the sub-apical cells of Ppsl1 Ppsl2 double mutants were almost identical in cell length whereas the wild type exhibited gradual increase of cell length along the filaments. This suggests that the gradual transition from chloronema to caulonema is regulated by class 1RSL gene function.

The hypothesis that class 1 RSL regulates gradual transition from chloronema to caulonema in protonema development was also tested by characterizing the duration of the cell cycle along protonemal filaments. If all cells in the Ppsl1 Ppsl2 double mutants are chloronema, it would be expected that there would be no gradual decrease in cell cycle times throughout the development of Ppsl1 Ppsl2 mutant protonemal filaments. As expected, cells in Ppsl1 Ppsl2 double mutants divided approximately every 24 hours (table 3.1) and the total cell number was increased by one per day; 3, 4, 6, 8 and 10 day old protonemal filaments of Ppsl1 Ppsl2 contained 3 ± 0.56 (S.D.), 3.91 ± 0.49 (S.D.), 5.57 ± 0.49 (S.D.), 7.87 ± 0.60 (S.D.), and 10 ± 0.86 (S.D.) cells. Together this data indicates that gradient cell growth along the filament is caused by a gradual transition from chloronema to caulonema which are regulated by the class 1 RSL gene function.

3.3.4 Class 1 RSL genes are expressed in protonema

To demonstrate that PpRSL1 and PpRSL2 control the transition from chloronema to caulonema in protonema development, transcriptional expression of class 1 RSL was tested in developing protonema by RT-PCR. PpRSL1 and PpRSL2 were expressed in protonemal stage. Furthermore, transcriptional expression levels of PpRSL1 gene in wild type was
almost identical to transcriptional expression levels of \textit{PpRSL1} in \textit{Pprsl2} single mutant, and transcriptional expression levels of \textit{PpRSL2} gene was also identical to transcriptional expression levels of \textit{PpRSL2} in \textit{Pprsl1} single mutants. (figure 3.4A). This indicates that \textit{PpRSL1} does not regulate \textit{PpRSL2} expression and \textit{PpRSL2} does not regulate \textit{PpRSL1} expression.

\textbf{3.3.5 \textit{PpRSL1} is strongly expressed in proximal regions of protonema}

In \textit{A. thaliana} class 1 \textit{RSL} genes are expressed in trichoblasts but not in root hair cells, suggesting class 1 \textit{RSL} regulates root hair differentiation from the hair forming epidermal cells (Menand et al., 2007b). If class 1 \textit{RSL} gene functions between caulonema differentiation in \textit{P. patens} and root hair differentiation in \textit{A. thaliana} have been conserved during land plant evolution, it was expected that \textit{PpRSL1} and \textit{PpRSL2} would be expressed in chloronema cells which develop into caulonema. To determine class 1 \textit{RSL} expression pattern in developing protonema spatial expression pattern of \textit{PpRSL1} and \textit{PpRSL2} was analysed using GUS expression system. For the GUS staining analysis, \textit{PpRSL1}_{\text{promoter-}}GUS and \textit{PpRSL2}_{\text{promoter-}}GUS fusions constructs were generated and introduced into the endogenous \textit{PpRSL1} and \textit{PpRSL2} loci by homologous recombination. The expression of \textit{PpRSL1}_{\text{promoter-}}GUS showed that \textit{PpRSL1} expressed in a gradient along protonemal filaments. \textit{PpRSL1} expression was detected in proximal chloronema of filaments at the centre of colonies by 8 hours after incubation. With increase of incubation time the \textit{PpRSL1} expression extended out to distal region of protonema. However, \textit{PpRSL1} expression was not detected in the distal caulonema cells (figure 3.4B, C, D). \textit{PpRSL2} expression was much less specific than \textit{PpRSL1}. \textit{PpRSL2} expressed not only in the proximal region of filaments but also in cells of distal region (figure 3.4E, F, G). Together
Figure 3. *PpRSL1* and *PpRSL2* genes are expressed in protonema

A. RT-PCR demonstrates steady state levels of *PpRSL1* and *PpRSL2* mRNA in protonema.

B-D. Time course of *PpRSL1* promoter::GUS expression in protonema.

E-G. Time course of *PpRSL2* promoter::GUS expression in protonema.

Scale bars = 100 µm.
these data indicate that \textit{PpRSL1} and \textit{PpRSL2} are expressed in protonema and \textit{PpRSL1} is expressed most highly in proximal chloronema.

3.3.6 Auxin positively regulates \textit{PpRSL1} and \textit{PpRSL2} expression

Because class 1 \textit{RSL} genes regulate root hair differentiation independently of auxin in \textit{A. thaliana}, it was expected that the class 1 \textit{RSL} would regulate caulonema differentiation independently of auxin if regulatory interaction between auxin and class 1 \textit{RSL} genes are conserved between these two species. The hypothesis was tested by carrying out RT-PCR using mRNA that was isolated both from auxin-treated and untreated wild-type protonema. Auxin treatment increased the transcriptional expression levels of \textit{PpRSL1} and \textit{PpRSL2}; both 20 hour treatment with 10 µM NAA and 14-day treatment with 1µM NAA induced the increase of transcriptional expression levels of \textit{PpRSL1} and \textit{PpRSL2} mRNA compared with the untreated control (figure 3.5A). This indicates that auxin positively regulates the transcriptional expression of both class 1 \textit{RSL} genes.

To confirm individually that auxin positively regulates transcriptional expressions of \textit{PpRSL1} and \textit{PpRSL2}, GUS staining assay using \textit{PpRSL1/promoter:GUS} and \textit{PpRSL2/promoter:GUS} was carried out. NAA-treatment increased GUS expression both in \textit{PpRSL1/promoter:GUS} and \textit{PpRSL2/promoter:GUS} plants compared with untreated controls. Auxin treatment induced the change of the \textit{PpRSL1} expression pattern; the expression of the \textit{PpRSL1/promoter:GUS} extended out to the apical tips of the protonemal filaments in NAA-treated condition, whereas the \textit{PpRSL1} expression was undetectable in these distal regions in the NAA-untreated controls (figure 3.5B, C). This indicates that auxin regulates the expression level and the spatial expression pattern of \textit{PpRSL1} in protonema filaments.
Figure 3.5 Auxin positively regulates *PpRSL1* and *PpRSL2* expression

A. RT-PCR showing that auxin increases steady state levels of *PpRSL1* and *PpRSL2* mRNA. On the left, short term effect of 10 μM auxin treatment for 20 hours. On the right, long term effect of 1 μM auxin treatment for 14 days.

B. Expression of *PpRSL1* promoter:GUS in control conditions (no NAA-treatment).

C. Expression of *PpRSL1* promoter:GUS after auxin treatment (1 μM NAA, for 10 days).


E. Expression of *PpRSL2* promoter:GUS after auxin treatment (1 μM NAA, for 10 days).

Scale bars = 100 μm.
Auxin-treatment also induced increase of PpRSL2promoter :GUS expression (figure 3.5D,E). However, auxin treatment did not induce significant change in the spatial expression pattern of PpRSL2.

3.3.7 Auxin-induced caulonema differentiation requires class 1 RSL gene activity

Because auxin positively regulates the expression of class 1 RSL in protonema, this supports the hypothesis that auxin regulates caulonema differentiation by positively regulating PpRSL1 and PpRSL2 expression in developing protonema. If this is true, Pprsl1 Pprsl2 double mutants would be resistant to auxin treatment. The hypothesis was supported by testing auxin effect on caulonema differentiation in wild type, Pprsl1 single, Pprsl2 single mutant and Pprsl1 Pprsl2 double mutant plants. Auxin treatment of wild type, Pprsl1 single and Pprsl2 single mutants developed extensive growth of caulonema and suppressed side branching formations, which resulted in the loose protonema in density compared with untreated controls (figure 3.6). However, Pprsl1 Pprsl2 double mutants were resistant to auxin (figure 3.7). Morphology of Pprsl1 Pprsl2 protonema between auxin-treated and untreated controls was almost identical; Pprsl1 Pprsl2 double mutant plants did not differentiate caulonema cells even in auxin-treated condition. These results indicate that auxin controls the caulonema differentiation by positively regulating PpRSL1 and PpRSL2 expression. In A. thaliana, auxin regulates root hair differentiation independently of class 1 RSL. Interestingly, this indicates that the regulation of class 1 RSL expression by auxin in P. patens does not exist in A. thaliana.
Figure 3. 6 Images showing *Pprsl1* and *Pprsl2* single mutants are not resistant to auxin.

A. Wild type protonema grown on solid minimal media for 2 weeks
B. Wild type protonema grown on solid minimal media including 1µM NAA for 2 weeks
C. *Pprsl1* single mutant protonema grown on solid minimal media for 2 weeks
D. *Pprsl1* single mutant protonema grown on solid minimal media including 1µM NAA for 2 weeks
E. *Pprsl2* single mutant protonema grown on solid minimal media for 2 weeks
F. *Pprsl2* single mutant protonema grown on solid minimal media including 1µM NAA for 2 weeks

Scale bars = 500 µm
Figure 3. 7 Images showing *Pprsl1Pprsl2* protonema is resistant to auxin.

A. Wild type protonema grown on solid minimal media for 2 weeks
B. Distal region of the filament of A
C. Wild type protonema grown on solid minimal media including 1µM NAA for 2 weeks
D. Distal region of the filament of C
E. *Pprsl1 Pprsl2* double mutant protonema grown on solid minimal media for 2 weeks
F. Distal region of the filament of E
G. *Pprsl1 Pprsl2* double mutant protonema grown on solid minimal media including 1µM NAA for 2 weeks
H. Distal region of the filament of G

Scale bars = 500 µm in A, C, E and G; 100 µm in B, D, F and H.
3.3.8 Proximal chloronema cells are sensitive to auxin

Since auxin positively regulates \textit{PpRSL1} and \textit{PpRSL2} expression in developing protonema and these genes are expressed strongly in proximal chloronema, it was expected that proximal chloronema would accumulate a relatively high concentration of auxin compared with distal caulonema cells. Since it was almost impossible to analyze directly the auxin concentration accumulated in each filamentous cell, the \textit{GmGH3} promoter activity that is highly responsive to auxin was analysed. Protonemal filaments transformed with \textit{GmGH3} \textit{promoter:GUS} do not express \textit{GUS} gene expression in auxin-untreated condition (figure 3.8; Bierfreund et al., 2003). Instead, 1µM NAA treatment induced \textit{GUS} gene expression in the cells of proximal regions by 2 hours after incubation (figure 3.8B). With the increase of incubation time the \textit{GUS} expression extended out to the distal region of protonema (figure 3.8C). The \textit{GUS} expression pattern in the \textit{GmGH3} \textit{promoter:GUS} was individually confirmed by 0.1 µM NAA treatment. GUS gene expression was detected in the first proximal cells in 0.1 µM NAA-treated conditions within 2 hours of incubation (figure 3.8E). With the increase of incubation time, \textit{GUS} expression gradually extended out to the cells in the distal region (figure 3.8F). This suggests that a relatively high concentration of auxin is accumulated in proximal chloronema cells and the auxin concentration decreases in apical caulonema cells. Furthermore, the \textit{GUS} expression patterns between \textit{GmGH3} \textit{promoter:GUS} and \textit{PpRSL1} \textit{promoter:GUS} plants were similar. These results support that auxin regulates caulonema differentiation by positively regulating class 1 \textit{RSL} in protonema development.

3.3.9 Class 1 \textit{RSL} gene expression is sufficient for caulonema differentiation from chloronema
Figure 3. 8 Images showing proximal chloronema cells are sensitive to auxin

**A-C.** Expression pattern of $GmGH3_{\text{promoter}:GUS}$ grown on minimal media including 1 µM NAA for 1 week in different incubation time.

**D-F.** Expression pattern of $GmGH3_{\text{promoter}:GUS}$ grown on minimal media including 0.1 µM NAA for 1 week in different incubation time.

Scale bars = 100 µm
Because auxin promotes caulonema differentiation by positively regulating the expression of both \textit{PpRSL1} and \textit{PpRSL2}, it was predicted that overexpression of class 1 \textit{RSL} genes would display similar morphology to wild type plants grown in auxin-treated conditions. To test this hypothesis \textit{35S:PpRSL1}, \textit{35S:PpRSL2} and \textit{35S:PpRSL1;35S:PpRSL2} plants were generated. Plants transformed with either \textit{35S:PpRSL1} or \textit{35S:PpRSL2} constructs were indistinguishable from the wild type in protonema development (figure 3.9). This suggests that neither expression of \textit{PpRSL1} nor \textit{PpRSL2} individually is sufficient for caulonema differentiation. Instead, plants transformed with \textit{35S:PpRSL1;35S:PpRSL2} strongly activated caulonema differentiation and did not develop a gradient cell identity along filaments (figure 3.10). This data supports the hypothesis that auxin promotes caulonema development by positively regulating \textit{PpRSL1} and \textit{PpRSL2} expression.

\section*{3.3.10 Involvement of polar auxin transporting system in regulating caulonema differentiation in protonema development}

TIBA inhibits polar auxin transport in moss filaments (Rose et al., 1983a). If polar auxin transport regulates caulonema differentiation in \textit{P. patens}, it was predicted that TIBA or NPA treatment would have an effect upon caulonema differentiation in protonema development. To test the hypothesis TIBA and NPA effect on caulonema differentiation was tested. Both TIBA and NPA treatment of the wild type protonema strongly suppressed caulonema differentiation in protonema development (figure 3.11). The TIBA effect on caulonema differentiation was characterized by measuring filamentous cell lengths. TIBA treatment induced the decrease of sub-apical cell lengths while first cell lengths in proximal regions between TIBA treated and untreated wild type protonema were almost same. Furthermore, the first cells in proximal regions and sub-apical cells are almost
Figure 3. Images showing plants transformed with either 35S:PpRSL1 or 35S:PpRSL2 construct are identical to wild type
A. Wild type protonema grown for 3 weeks
B. Protonema transformed with 35S:PpRSL1 3 weeks after plating
C. Protonema transformed with 35S:PpRSL2 3 weeks after plating
Scale bars = 100 µm
Figure 3. 10 Caulonema differentiation is constitutively activated in protonema transformed with $35S$:PpRSL1;$35S$:PpRSL2

A. Wild type colonies 7 days after plating
B. Wild type colonies 21 days after plating
C. Protonema transformed with $35S$:PpRSL1;$35S$:PpRSL2 grown for 7 days
D. Protonema transformed with $35S$:PpRSL1;$35S$:PpRSL2 grown for 21 days
E. Relationship between cell length and position along proximal-distal axis of 7-day old protonemal filaments. Asterisk indicates significant difference between wt and transgenic plants based on 95% confidence intervals. Scale bars = 100 μm.
Figure 3. TIBA treatment inhibits caulonema differentiation in protonema of wild type.
A. 7 day old wild type protonema grown on normal condition.
B. 7 day old wild type protonema grown on TIBA-treated condition.
C. 7 day old wild type protonema grown on NPA-treated condition.
D. Change First and sub-apical cell lengths between TIBA treated and untreated control.
Asterisk indicates significant difference based on 95 % confidence intervals.
Scale bars = 100 μm.
identical in cell lengths, suggesting that polar auxin transport is involved in caulonema differentiation in protonema development.

3.3.11 Pprsl1Pprsl2 and 35S:PpRSL1;35S:PpRSL2 plants are resistant to TIBA

Because class 1 RSL genes regulate caulonema differentiation downstream of auxin, it would be expected that mutant plants which lack both PpRSL1 and PpRSL2 functions and 35S:PpRSL1;35S:PpRSL2 transgenic plants would be resistant to TIBA. To test the hypothesis, the TIBA effect on protonema development of Pprsl1Pprsl2 double mutants and 35S:PpRSL1;35S:PpRSL2 transgenic plants was determined. As expected, Pprsl1Pprsl2 double mutants are resistant to TIBA (figure 3.12A, B). TIBA treatment did not cause a morphological change of the Pprsl1Pprsl2 protonema. Similarly, 35S:PpRSL1;35S:PpRSL2 transgenic plants were also resistant to TIBA. Plants transformed with 35S:PpRSL1;35S:PpRSL2 differentiated caulonema in TIBA-treated conditions (figure 3.12E, F, I, J). This data also indicates that class 1 RSL regulates caulonema differentiation downstream auxin.

3.4 Discussion

3.4.1 Class 1 RSL regulate differentiation of the specialized tip growing cells required for nutrient uptake

Plants differentiate specialized cell types for nutrient uptake. Rhizoids in non vascular plants and root hairs in vascular plants differentiate as specialized cells for nutrient uptake
Figure 3. 12 Images showing *Pprsl1 Pprsl2* and *35S:PpRSL1;35S:PpRSL2* plants are resistant to TIBA.

A. 7 day old *Pprsl1 Pprsl2* protonema grown on minima media

B. 7 day old *Pprsl1 Pprsl2* protonema grown on minimal media including 100 µM TIBA

C. 3 week old wild type protonema grown on minima media

D. Distal region of the filament of C

E. 3 week old *35S:PpRSL1;35S:PpRSL2* protonema grown minima media

F. Distal region of the filament of E

G. 3 week old wild type protonema grown on minimal media including 100 µM TIBA

H. Distal region of the filament of G

I. 3 week old *35S:PpRSL1;35S:PpRSL2* protonema grown on minimal media including 100 µM TIBA

J. Distal region of the filament of I. Scale bars = 100 µm.
(Gahooonia et al., 1997; Duckett et al., 1998). Protonema is the first phase in the haploid stage of the moss life cycle. Protonema does not develop rhizoids which develop on gametophores. Instead, it has been hypothesised that caulonema are the specialized cells which function in nutrient uptake and transport during protonema development. This hypothesis was supported by observations that caulonema and rhizoid differentiation are regulated by nutrient conditions with the same regulation pattern (Goode et al., 1992). Furthermore, the morphological resemblance between caulonema and rhizoids also supports that caulonema differentiate as specialized cells for nutrient uptake (Duckett et al., 1998).

In this study it has been shown that class 1 \textit{RSL} regulates caulonema differentiation from chloronema in growing protonema. Class 1 \textit{RSL} genes control the differentiation of caulonema and rhizoids in \textit{P. patens}, and root hairs in \textit{A. thaliana} (Masucci and Schiefelbein, 1994; Menand et al., 2007b). Although these cells grow by tip-growth, not all tip growing cell differentiations are controlled by class 1 \textit{RSL} gene functions. For example, chloronema cells which mainly function in photosynthetic assimilation in protonema development grow by tip growth, but development of chloronema is not regulated by class 1 \textit{RSL} gene functions in developing protonema; chloronema cells which develop from wild type protonema and \textit{Pprsl1 Pprsl2} protonema are morphologically identical. Similarly, the development of tip-growing pollen tubes does not require \textit{RSL} gene function in \textit{A. thaliana} (Menand et al., 2007b). These results indicate that the \textit{RSL} genes are not simply positive regulators to control differentiation and development of tip-growing cells. Instead \textit{RSL} genes control the differentiation of specialized tip-growing cells that form at the interface of the plant and its substratum and are likely to have roles in nutrient uptake and anchorage.
3.4.2 Gradient expression of \textit{PpRSL1} in protonema development regulates gradient cell growth along filaments

In this study it has been shown that the gradual transition from chloronema to caulonema results in the gradient cell identity along wild type filaments; the first cells to differentiate from germinating spores are chloronemal and by 4 days after germination, new cells which are produced by apical cell division at the growing edge of colonies become progressively more caulonemal in character. Class 1 \textit{RSL} genes regulate the developmental transition from chloronema to caulonema in protonema development. \textit{PpRSL1} is most highly expressed in the proximal chloronema cells. The expression level of \textit{PpRSL1} progressively decreases as cells become more caulonemal in character and apical caulonema in distal regions do not express \textit{PpRSL1}, suggesting that the gradient cell growth along wild-type filaments is caused by the gradient expression of \textit{PpRSL1}.

In this study, I showed moss class 1 \textit{RSL} regulates caulonema differentiation from chloronema; \textit{Pprsl1 Pprsl2} double mutant plants develop only chloronema along filaments and \textit{35S:PpRSL1;35S:PpRSL2} overexpressing plants activate caulonema differentiation from chloronema. Despite the fact that \textit{PpRSL1} positively regulates caulonema development this gene is predominantly expressed in proximal chloronema cells. Therefore \textit{PpRSL1} appears to be expressed in the wrong place! I think that two possible mechanisms can explain this. The first hypothesis is that class 1 RSL proteins move along filaments. It is possible that \textit{PpRSL1} gene is transcribed in proximal cells of the protonema and translated. Then the PpRSL1 proteins could move from proximal cells to distal cells and accumulate highly in distal chloronema, where it is active in promoting caulonema development. To test the hypothesis we generated GFP-fused RSL expression system.
Unfortunately, GFP signals were not detected in the plants transformed with N and C-term GFP-fused RSL genes (data not shown). Therefore, some alternative approaches including a combination of immunolocalization of protein and in situ hybridization of mRNA are required for proving the hypothesis. However, it is likely that proteins movement is not required for the function of RLS genes in *A. thaliana* because the transcriptional and translational expression pattern of class 1 RSL is almost identical in *A. thaliana* (Menand et al., 2007; Yi et al., 2010). The second hypothesis is that gradual accumulations of some factors downstream of class 1 RSL directly regulate caulonema differentiation from chloronema. Since the downstream of class 1 *PpRSL* is poorly understood, it is hard to test this hypothesis, now. In this respect, I think that further studies about downstream components of class 1 RSL related to caulonema development are required for expanding our understanding of rooting cell development and evolution.

### 3.4.3 Regulatory relationship between auxin and class 1 RSL in *Physcomitrella patens* is different from *Arabidopsis thaliana*

In this study, it has been shown that auxin regulates caulonema differentiation by positively regulating class 1 RSL genes in *P. patens*; auxin treatment increases the transcriptional expression level of class 1 RSL, and *Pprsl1Pprsl2* double mutants are resistant to auxin. Furthermore, the spatial pattern of *GmGH3* promoter::GUS is similar to the expression pattern of *PpRSL1*, suggesting that the *PpRSL1* gene is strongly expressed in cells which accumulate a relatively high concentration of auxin. In contrast to *P. patens*, class 1 RSL of *A. thaliana* regulates root hair differentiation independently of auxin; auxin treatment does not increase the transcriptional expression level of class 1 RSL of *A. thaliana*, and *Atrhd6 Atrsl1* double mutants are not resistant to auxin (Yi et al., 2010).
Furthermore, class 1 RSL genes are strongly expressed in trichoblasts which accumulate a relatively low concentration of auxin (Jones et al., 2009). These results suggest that even though caulonema and root hair differentiation requires both class 1 RSL function and auxin action, it is likely that the interaction between them has changed during land plant evolution.

Although the spatial pattern of $GmGH3_{promoter}$:GUS in protonema suggests that relatively high concentration of auxin is accumulated in proximal chloronema compared to distal caulonema cells, it is still possible that different cell sensitivity to auxin between proximal chloronema and distal caulonema cells might results in the spatial expression pattern of $GmGH3_{promoter}$:GUS. If it is assumed that endogenous auxin concentrations of proximal chloronema and distal caulonema cells are almost identical, and the proximal cells are more sensitive to auxin than distal caulonema cells, the proximal chloronema cells would show relatively strong auxin response compared to distal caulonema cells, as observed. Therefore, the spatial expression pattern of $GmGH3_{promoter}$:GUS alone cannot distinguish between these two possibilities.

An understanding of the pattern of polar auxin transport in developing caulonema could distinguish between these two alternatives. However, the pattern of polar auxin transport in moss protonema development has not been well studied. Previous studies using moss rhizoid system showed that proximal rhizoid cells accumulate relatively high concentrations of auxin compared to distal rhizoid cells; when whole rhizoid of mosses were incubated on an agar containing $[^{14}\text{C}]-\text{IAA}$, the radiolabel IAA was strongly detected in proximal rhizoid cells compared to distal rhizoid cells (Rose and Bopp, 1983). This result indicates that auxin moves from distal rhizoid cells to proximal rhizoid cells. If
protonemal filaments and rhizoid filaments use the same polar auxin transport system for their developments, it is expected that polar auxin transport from distal caulonema cells to proximal chloronema leads to accumulation of auxin in proximal cells. Although this model could explain the spatial expression pattern of \( GmGH3\text{promoter:GUS} \) during protonema development, there is still a need for further studies to demonstrate that the basipetal transport of auxin occurs in protonema as well as rhizoids. Therefore, characterization of polar auxin transport along protonemal filaments using \([^{14}\text{C}]-\text{IAA}\) is important. If proximal cells accumulate higher levels of \([^{14}\text{C}]-\text{IAA}\) than distal cells, it is expected that auxin moves from distal region to proximal region in protonema development.

The mechanism underpinning polar auxin transport in \( P.\ patens \) remains to be characterized. In \( \text{Arabidopsis} \), PIN-mediated auxin transport system has been well studied and showed that the polar auxin transport generates auxin gradients and regulates plant cell development (Vanneste and Friml, 2009). \( \text{Arabidopsis} \) genome contains eight \( \text{PIN} \) genes, which can be classified into two major types, plasma membrane-localized PIN1 type and endoplasmic reticulum (ER)-localized PIN5 type; plasma membrane PIN proteins regulate cell-to-cell auxin movement while ER PIN proteins control intracellular auxin homeostasis (Mravec et al., 2009). Three PIN-like genes were identified in \( P.\ patens \) based on protein sequence similarities. Moss PIN proteins are located in the ER, suggesting ER-localized PIN proteins appeared early in land plant evolution (Mravec et al., 2009). Since ER-located PIN proteins are unlikely to be involved in cell to cell movement of auxin, the identified PIN proteins from \( P.\ patens \) cannot account for the polar transport of auxin movement in rhizoid filaments (Rose and Bopp, 1983) and TIBA/NPA effect on
protonema development in this study. Therefore, more study on the precise mechanism of polar auxin transport in *P. patens* is required.

3.4.4 Possible involvement of class I *RSL* genes in determination of side branch-forming site in protonema development

The first cells which differentiate from germinating spores are chloronemal. As filaments grow, some chloronemal apical cells develop into caulonema cells. The sub-apical caulonema usually produce side branch cells (Cove, 2005). Initials of side branching usually form at the distal end of the filamentous cells in wild type protonema (figure 3.13). It is unknown which factors are involved in the determination of side branch forming sites in protonema development. Interestingly, protonemal filaments transformed with 35S:*PpRSL1;*35S:*PpRSL2, developed abnormal side branching formations in protonemal development, with relatively high frequency and strength compared with wild type; some protonemal filaments of 35S:*PpRSL1;*35S:*PpRSL2 developed side branches in the middle of filamentous cells (figure 3.13). Although wild type filaments sometimes develop abnormal side branches, the frequency and strength are much lower than plants transformed with 35S:*PpRSL1;*35S:*PpRSL2. Interestingly, the *AtRHD6* gene is also involved in the determination of root hair forming sites in *A. thaliana*; root hairs of *Atrhd6* mutants form more basally on the hair forming epidermal cells while root hairs of wild type develop from the distal end of epidermal cells (Schiefelbein and Somerville, 1990; Masucci and Schiefelbein, 1994). Despite the structural difference between multi-cellular side branches and single cellular root hairs, this suggests that class 1 *RSL* might be involved in the determination of the side branch forming sites in *P. patens*. However, the abnormality in the side branch forming sites was not observed in protonemal filaments of
Figure 3. Images showing plants transformed with 35S:PpRSL1;35S:PpRSL2 developed abnormal side branching formations in protonemal development with relatively high frequency and strength compared with wild type (A and B: wild type protonemal filaments, C-F: Plants transformed with 35S:PpRSL1;35S:PpRSL2) Protonemal filaments usually developed side branch formation at the distal end of filamentous cells (A). Sometimes, wild-type filaments formed side branches more basally on filamentous cells (B). Plants transformed with 35S:PpRSL1;35S:PpRSL2 usually developed side branch formation at the distal end of filamentous cells like wild type (C). However, plants transformed with 35S:PpRSL1;35S:PpRSL2 developed abnormal side branching formations in location with relatively high frequency and strength compared with wild type (D, E, F). Arrows and arrowheads indicate side branch initials and cell walls, respectively. Scale bar = 100 μm
double mutants. The possible involvement of class 1 RSL in the determination of side branch-forming sites needs more studies.

3.4.5 Possible interaction between PpRSL1 and PpRSL2 protein in regulating caulonema differentiation of P. patens

Basic Helix-Loop-Helix (bHLH) transcription factors are involved in cell specification and differentiation during animal and plant cell development (Molkentin et al., 1996). It has been reported that bHLH proteins bind to DNA as heterodimers although they, usually more weakly, bind to DNA as homodimers (Murre et al., 1989). Since both PpRSL1 and PpRSL2 are expressed in developing protonema it has been suggested that PpRSL1 and PpRSL2 form heterodimers and regulate caulonema differentiation. Furthermore, in vitro pull-down assay using His-tagged PpRSL1 and GST-tagged PpRSL2 showed PpRSL1 interacts with PpRSL2 in E. coli system (figure 3.14). This possible interaction between PpRSL1 and PpRSL2 needs to be confirmed by in vivo binding assay such as co-immunoprecipitation. Nevertheless it is consistent with the hypothesis that overexpression results in an increase in the amount of PpRSL1-PpRSL2 heterodimers. Given that co-overexpression of PpRSL1 and PpRSL2 strongly activated caulonema differentiation while overexpression of PpRSL1 and PpRSL2 individually has no effects on morphology, these data suggest that PpRSL1 and PpRSL2 act together in heterodimers. Taken together these data support the hypothesis that the PpRSL1-PpRSL2 heterodimer is the most active form that activates caulonema differentiation during protonema development.
Figure 3.14. PpRSL1 interacts with PpRSL2. In vitro pull down assay of His-tagged PpRSL1 and GST-fused PpRSL2 protein was carried out. Crude extracts of His-tagged and GST-fused PpRSL2 protein were incubated for 3 hr at 4°C. Proteins purified with Ni-NTA resins were immunoblotted with GST antibody.
Chapter Four:

Class 1 RSL function

in rhizoid differentiation

of *Physcomitrella patens*
4.1 Summary

Rhizoids are the filamentous rooting cells which develop on leafy shoot gametophyte (gametophores). Two kinds of rhizoids, basal and mid-stem rhizoids, can be distinguished by their developing positions in *P. patens*. Auxin and class 1 *RSL* genes encoding basic helix loop helix transcription factors regulate rhizoid differentiation in *P. patens*. However, the regulatory interaction between auxin and class 1 *RSL* is unknown. In this study, it has been shown that class 1 *RSL* genes regulate the differentiation of basal and mid-stem rhizoids from specialized cells which give rise to rhizoids. Auxin regulates rhizoid differentiation by positively regulating class 1 *RSL* genes; transcriptional expression levels of the class 1 *RSL* genes are increased by auxin treatment and mutant plants which lack class 1 *RSL* (*Pprsl1 Pprsl2*) are resistant to auxin. The spatial patterns between class 1 *RSL* expression and auxin distribution in developing gametophores are almost identical. Furthermore, co-expression of *PpRSL1* and *PpRSL2* is sufficient for activating rhizoid differentiation in the absence of auxin. These results suggest that the positive regulation of class 1 *RSL* by auxin controls rhizoid differentiation in *P. patens*. In contrast to *P. patens*, auxin regulates root hair differentiation independently of class 1 *RSL* in *A. thaliana*. This indicates that even though rhizoid and root hair differentiation requires the conserved function of class 1 *RSL*, the regulation of class 1 *RSL* by auxin is different in these two plants.
4.2 Introduction

Rhizoids are filamentous rooting cells in non vascular plants; they function in anchoring plants in substratum and in acquiring nutrients (Duckett et al., 1998). These functions in non vascular plants are the same as the functions of root hairs in vascular plants (Peterson and Farquhar, 1996; Gahoonia et al., 1997). In *P. patens*, protonemal colonies develop buds on the protonemal filaments and the buds develop into gametophores, leafy gametophyte. Rhizoids form on gametophores. The morphology of rhizoids is very similar to caulonema; they have poorly developed chloroplasts and oblique cell walls. The brown pigmentation in rhizoids is the most distinguishable trait from caulonema (Sakakibara et al., 2003) at the morphological level.

The gametophores of *P. patens* develop two types of rhizoids, basal rhizoids and mid-stem rhizoids. The two types of rhizoids in *P. patens* can be distinguished by the position in which the rhizoids develop on the gametophores; basal rhizoids form in the basal regions of the gametophore below juvenile leaves, while mid-stem rhizoids develop in more apical regions below adult leaves (Sakakibara et al., 2003). The basal rhizoids differentiate from any epidermal cells near the base of the young gametophores, whereas mid-stem rhizoids differentiate from the epidermal cells which are located to the outside of two small leaf trace cells just below the adult leaves.

Auxin positively regulates rhizoid differentiation in developing gametophores. Auxin treatment of wild type plants strongly increases the number of both basal and mid-stem rhizoids (Ashton et al., 1979; Sakakibara et al., 2003). Mutant plants which lack the *PpIAA*
encoding moss Aux/IAAs develop none or few rhizoids in their gametophores (Prigge et al., 2010), indicating that auxin is required for rhizoid differentiation in *P. patens*. Auxin controls rhizoid differentiation by regulating the expression of downstream regulators which are required for rhizoid differentiation. For example, the Auxin-responsive *PpHb7* gene encoding an homeodomain-leucine zipper I (HD-Zip I) subfamily protein is required for the brown pigmentation in rhizoid development. Mutants that lack *PpHb7* function failed to accumulate brown pigments in rhizoids. However the mutant plants have no defects in rhizoid differentiation except for the brown pigmentation (Sakakibara et al., 2003). Therefore, it is likely that auxin positively regulates the expression of key regulators required for rhizoid differentiation in developing gametophores.

*PpRSL1* and *PpRSL2* which belong to class 1 RSL in *P. patens* are basic-helix-loop-helix transcription factors that are required for the rhizoid differentiation; mutants that lack the *PpRSL1* and *PpRSL2* functions develop few very short basal rhizoids, indicating that *PpRSL1* and *PpRSL2* regulate rhizoid development (Menand et al., 2007b). Nevertheless, the regulatory relationship between *PpRSL1* and *PpRSL2* and auxin is unknown.

This study has shown that both basal and mid-stem rhizoid differentiation in *P. patens* requires *PpRSL1* and *PpRSL2* expression which is positively regulated by auxin. Furthermore, the *PpRSL1 PpRSL2* double mutants are resistant to auxin and co-expression of *PpRSL1* and *PpRSL2* is sufficient to activate rhizoid differentiation in developing gametophores. Together this data suggests that auxin regulates rhizoid differentiation by positively regulating expression of class 1 RSL genes in *P. patens*. 
4.3 Results

4.3.1 *PpRSL1* and *PpRSL2* are required for the development of both basal and mid-stem rhizoids.

Two types of rhizoids, basal and mid-stem rhizoids develop on the gametophores of *P. patens*. Basal rhizoids develop at the gametophore base from early gametophore development. On the other hand, mid-stem rhizoids which develop in more apical regions below adult leaves differentiate from specialized epidermal cells, which are located to the outside of two small leaf trace cells which extend from the stem into the midrib of the adjoining adult leaves. These two types of rhizoids are morphologically identical and can be distinguished by the position on the gametophore where they develop (Sakakibara et al., 2003). To understand the function of class 1 *RSL* genes in rhizoid development, phenotypic analyses were carried out using *Pprs11, Pprs12* single and *Pprs11Pprs12* double mutants. *Pprs11* and *Pprs12* single mutants did not display morphological differences in rhizoid development compared to wild type. Both basal and mid-stem rhizoids develop in *Pprs11* and *Pprs12* single mutants (figure 4.1). On the other hand, only few very short basal rhizoids and no mid-stem rhizoids developed in *Pprs11 Pprs12* double mutants, indicating that both the *PpRSL1* and *PpRSL2* genes positively regulate both basal and mid-stem rhizoid differentiation in *P. patens*.

4.3.2 *PpRSL1* and *PpRSL2* are expressed in rhizoid forming cells

The class I *RSL* gene in *A. thaliana* controls root hair differentiation from trichoblasts. The function of class 1 *RSL* was supported by its expression pattern; *AtRHD6* and *AtRSL1* are expressed in the specialized epidermal cells that give rise to root hairs but not in the root hair cell itself (Menand et al., 2007b). Because rhizoid differentiation requires the
Figure 4. 1 Images showing *PpRSL1* and *PpRSL2* are required for the formation of basal and mid-stem rhizoids

(A-D) Basal rhizoid development in wild type (A), *Pprsl1* mutant (B), *Pprsl2* (C) and *Pprsl1 Pprsl2* double mutant (D).

(E-H) Mid-stem rhizoid development in wild type (E), *Pprsl1* mutant (F), *Pprsl2* (G) and *Pprsl1 Pprsl2* double mutant (H).

Scale bars = 500 µm in A-D and 200 µm in E-H.
conserved function of class 1 RSL, it was expected that *PpRSL1* and *PpRSL2* would express in the specialized cells which give rise to rhizoids. To test the hypothesis, the plants transformed with *PpRSL1*promoter:*GUS* and *PpRSL2*promoter:*GUS* were used for the analysis of class 1 RSL expression patterns in gametophores. *PpRSL1* is highly expressed in buds which develop into gametophores (figure 4.2A-D and I-M). When buds develop into gametophores, *PpRSL1* was expressed at the base of the gametophore axis where basal rhizoids develop. The expression of *PpRSL1* was restricted to the base of the gametophores. The *PpRSL1* expression pattern was clearly visualized in mature gametophores. Mature gametophores also expressed *PpRSL1* at their base. In the middle of the gametophore axis, only rhizoid forming cells below adult leaves showed strong expression of *PpRSL1*, suggesting *PpRSL1* is expressed in the cells that give rise to rhizoids in *P. patens*. To confirm that *PpRSL1* expresses in the specialized epidermal cells which give rise to rhizoids in gametophore development, the spatial expression pattern of *PpRSL1* was visualized in transverse section (figure 4.2D, M). Epidermal rhizoid forming cells in the base of the gametophores displayed a stronger expression of *PpRSL1* than other epidermal cells. In mid-stem regions, only rhizoid forming epidermal cells which are located outside of two small leaf trace cells expressed *PpRSL1*. These results indicate that *PpRSL1* is specifically expressed in the rhizoid forming epidermal cells in gametophores.

The *PpRSL2* expression pattern was similar to the *PpRSL1* in bud and young gametophore development. *PpRSL2* was expressed in buds which develop into gametophores and expression continued during bud development (figure 4.2E-G). *PpRSL2* was mainly expressed at the base of the gametophore axis in young gametophores. Later in gametophore development, *PpRSL2* was highly expressed in the base of the gametophores. However, *PpRSL2* was also expressed throughout the gametophore axis. To confirm that
Figure 4. 2 Images showing *PpRSL1* and *PpRSL2* genes are expressed in cells that give rise to rhizoids

(A-D) *PpRSL1* promoter:*GUS* expression pattern in young gametophores.

(E-H) *PpRSL2* promoter:*GUS* expression pattern in young gametophores.

(I–M) *PpRSL1* promoter:*GUS* expression pattern in adult gametophores with mid-stem rhizoids.

(N–R) *PpRSL2* promoter:*GUS* expression pattern in adult gametophores with mid-stem rhizoids.

Transverse sections through the mid-stem region of gametophores demonstrate that *PpRSL1* (L, M) is specifically expressed in the specialised cells that differentiate as rhizoids and *PpRSL2* (Q, R) is expressed throughout the epidermis. Arrows highlight mid-stem rhizoid forming cells with high levels of expression; arrow heads indicate the positions of the small leaf trace cells.

Scale bars = 50 µm in M and R; 100 µm in A, B, C, D, E, F, G, H, J, K, L O, P and Q; 500 µm in I and N.
PpRSL2 expresses in the rhizoid forming epidermal cells the spatial expression pattern of PpRSL2 was also visualized in transverse section. PpRSL2 was strongly expressed in the rhizoid forming epidermal cells in the base of the gametophore axis (figure 4.2H, R). Rhizoid forming epidermal cells in the mid-stem regions also displayed strong expression of PpRSL2. However, PpRSL2 expression was less specific than PpRSL1 expression in gametophores. This data suggests that specific expression of class 1 RSL in rhizoid-forming cells regulates rhizoid differentiation in developing gametophores of Physcomitrella patens.

4.3.3 Auxin regulates expression of PpRSL1 and PpRSL2 in gametophores

Auxin positively regulates rhizoid development (Ashton et al., 1979; Sakakibara et al., 2003). Auxin treatment increases the number of rhizoid formations in gametophores. Because the expression of PpRSL1 and PpRSL2 is required for rhizoid differentiation the hypothesis that auxin is involved in the regulation of PpRSL1 and PpRSL2 expression in rhizoid differentiation was tested.

The auxin effect on PpRSL1 and PpRSL2 expression in gametophores by RT-PCR analysis was determined. Auxin-treatment increased the transcriptional expression levels of the PpRSL1 and PpRSL2 genes, indicating auxin positively regulates PpRSL1 and PpRSL2 gene expression (figure 4.3A). To confirm regulation of PpRSL1 and PpRSL2 by auxin, GUS expression in the plants transformed with PpRSL1 promoter:GUS and PpRSL2 promoter:GUS constructs was tested in auxin-treated condition. As expected, auxin-treatment increased the expression of each reporter gene (figure 4.3B). Furthermore, GUS expression in auxin-treated conditions was detected along whole rhizoid filaments whereas GUS expression was restricted in rhizoid-forming cells in auxin-untreated controls. This
Figure 4. 3 Images showing auxin positively regulates the transcriptional expression of *PpRSL1* and *PpRSL2* genes

A. RT-PCR analysis indicates that a one week auxin treatment (1 µM NAA) increased steady state levels of *PpRSL1* and *PpRSL2* in four week old gametophores.

B. 1 µM NAA treatment increases the expression levels of *PpRSL1_{promoter}:GUS* and *PpRSL2_{promoter}:GUS* compared to untreated controls.
indicates that auxin treatment not only increases the transcriptional expression levels of class 1 RSL genes but also changes their spatial expression patterns in gametophores.

4.3.4 Pprsl1 Pprsl2 double mutants are resistant to auxin

Because auxin treatment increases transcriptional expression levels of class 1 RSL genes, it was hypothesized that auxin regulates rhizoid development by positively controlling class 1 RSL genes. To test the hypothesis, the resistance of Pprsl1 single, Pprsl2 single and Pprsl1 Pprsl2 double mutants towards auxin was determined. Auxin treatment of the wild type strongly activated rhizoid differentiation, which resulted in the development of large numbers of rhizoids on the gametophores compared with auxin-untreated controls (figure 4.4). Pprsl1 and Pprsl2 single mutants were also sensitive to auxin; auxin treatment induced extensive growth of rhizoids like the wild type (figure 4.5). However, auxin treatment of Pprsl1 Pprsl2 double mutants did not have an effect upon rhizoid differentiation on gametophores. Consequently, the morphologies between auxin-treated and untreated gametophores of Pprsl1 Pprsl2 double mutants were almost identical (figure 4.4). This data suggests that auxin requires PpRSL1 and PpRSL2 activities for rhizoid differentiation in developing gametophores.

4.3.5 Rhizoid-forming cells are sensitive to auxin

Since auxin positively regulates PpRSL1 and PpRSL2 expression, it was hypothesized that the relatively high concentration of auxin would be accumulated in the rhizoid-forming cells which specifically express class 1 RSL genes compared with other cell types of gametophores. To test the hypothesis, auxin distribution in gametophores using plants
Figure 4. 4 Images showing *PpRSL1* and *PpRSL2* gene activity is required for the induction of rhizoid development by auxin

NAA treatment (0.2, 1 and 10 µM) for one week enhances rhizoid development in wild type compared to the untreated control (0 µM), but not in the *Pprsl1 Pprsl2* double mutant. (A-D) wild type; (E-H) *Pprsl1 Pprsl2* double mutant. (A, E) 0 µM NAA (control), (B, F) 0.2 µM NAA, (C, G) 1 µM NAA, (D, H) 10 µM NAA.

Scale bars = 500 µm.
Figure 4. 5 Images showing *Pprsl1* and *Pprsl2* single mutants are sensitive to auxin. *Pprsl1* mutants are in the top row and *Pprsl2* mutants are in the bottom row.

A. *Pprsl1* gametophore grown on NAA-untreated condition.

B. *Pprsl1* gametophore grown on 1 µM NAA-treated condition for 1 week.

C. *Pprsl2* gametophore grown on NAA-untreated condition.

D. *Pprsl2* gametophore grown on 1 µM NAA-treated condition for 1 week.

Scale bar = 500 µm.
transformed with $GmGH3_{promoter}:GUS$ was determined (Bierfreund et al., 2003; Ludwig-Müller et al., 2009; Fujita et al., 2008; Eklund et al., 2010a; Eklund et al., 2010b). The $GmGH3$ promoter was highly sensitive to auxin (figure 4.6); auxin treatment induced strong expression of the $GUS$ gene throughout the gametophore axis, whereas untreated controls showed strong expression of $GUS$ genes only at the base of gametophore axis, which is almost identical to the expression patterns of $PpRSL1_{promoter}:GUS$ and $PpRSL2_{promoter}:GUS$-transformed plants (figure 4.6). This result suggests that a relatively high concentration of auxin is accumulated at the base of gametophore axis where rhizoids form. The spatial expression pattern of $GmGH3_{promoter}:GUS$ was also visualized in transverse section. $GmGH3_{promoter}:GUS$ was strongly expressed in all epidermal cells that gave rise to basal rhizoids in young gametophores, suggesting all epidermal cells in the basal region of the gametophores can develop rhizoids. Later in gametophore development, $GmGH3_{promoter}:GUS$ was strongly expressed in the epidermal rhizoid forming cells that develop into mid-stem rhizoids (figure 4.6). These results suggest that class 1 RSL is specifically expressed in cells which accumulate relatively more auxin than other cells.

4.3.6 Class 1 RSL expression is sufficient for rhizoid differentiation in developing gametophores

Because auxin activates rhizoid differentiation by positively regulating $PpRSL1$ and $PpRSL2$, it was expected that constitutive expression of $PpRSL1$ and $PpRSL2$ would be sufficient to promote rhizoid differentiation in $P. patens$. To test this hypothesis $35S:PpRSL1$, $35S:PpRSL2$ and $35S:PpRSL1;35S:PpRSL2$ plants were generated. Plants transformed with either $35S:PpRSL1$ or $35S:PpRSL2$ constructs were indistinguishable from the wild type (figure 4.7). This suggests that neither expression of $PpRSL1$ nor
Figure 4. 6 Images showing the spatial pattern of GmGH3 promoter activity in gametophores.

(A, B) NAA treatment increases GmGH3 promoter activity in gametophores.
A. GmGH3 expression pattern in untreated controls.
B. GmGH3 expression pattern in 1 µM NAA -treated condition for 1 week.

(C, D) The spatial pattern of GmGH3 promoter activity in basal and mid-stem region of the gametophores.
C. GmGH3 promoter is active in most cells in the epidermis of basal region.
D. GmGH3 promoter activity is higher in the epidermal cells that give rise to rhizoids cells located near leaf trace cells (arrow) in the mid-stem region.

Scale bars = 500 µm in A and B; 100 µm in C and D.
Figure 4. 7 Images showing Overexpression of *PpRSL1* and *PpRSL2* genes individually is not sufficient to activate rhizoid differentiation.

A. Wild type gametophores grown on minimal solid media.
B. Gametophores transformed with 35S:*PpRSL1* grown on minimal solid media.
C. Gametophores transformed with 35S:*PpRSL2* grown on minimal solid media.
Scale bars = 500 μm.
PpRSL2 individually is sufficient for rhizoid development. Instead, plants transformed with 35S:PpRSL1;35S:PpRSL2 strongly activated rhizoid differentiation. Buds produced masses of mature rhizoids in plants overexpressing both PpRSL1 and PpRSL2, while in the wild type there were very few and immature rhizoids on the bud (figure 4.8). Only few gametophores developed in these cultures because most cells in the buds overexpressing both PpRSL1 and PpRSL2 differentiated into rhizoids (figure 4.8D, H); 42.07 ± 5.46 (standard deviation, n=10) gametophores.cm⁻² developed from wild type protonema whereas 0.68 ± 0.79 (standard deviation, n=12) gametophores.cm⁻² developed from protonema overexpressing both PpRSL1 and PpRSL2 (table 4.1). The few gametophores that formed in plants overexpressing both PpRSL1 and PpRSL2 also developed massive rhizoids compared with almost the same age of wild type gametophores.

If co-expression of PpRSL1 and PpRSL2 is sufficient to promoter rhizoid differentiation, the possibility that other types of cells such as cortex cells differentiate rhizoids in the plants transformed with 35S:PpRSL1;35S:PpRSL2, was tested. The internal cellular anatomy of 35S:PpRSL1;35S:PpRSL2 plants was visualized in transverse section. Despite the increase of rhizoid formation in both base and mid-stem epidermal cells, all rhizoids differentiate from epidermal cells (figure 4.8 I, J, K, L). The internal cellular anatomy of the Pprsl1 Pprsl2 mutant gametophores was also identical to the wild type in spite of the obvious defects in rhizoid development (figure 4.9). These data indicate that PpRSL1 and PpRSL2 are required for the differentiation of both basal and mid-stem rhizoids from specialized epidermal cells, which give rise to rhizoids but are not required for the development of other type of cells in the gametophores. These results suggest that the PpRSL1 and PpRSL2 genes specifically regulate rhizoid differentiation from epidermal cells.
Figure 4. 8 Images showing *PpRSL1* and *PpRSL2* genes expression is sufficient for rhizoid system development in *P. patens*

(A) Wild type gametophore bud that has just started to develop rhizoids but not yet begun to form leaves. (B) Young wild type gametophore with the first juvenile leaves. (C) Older gametophore than that shown in (B). (D) Three week old wild type protonema with developing leafy gametophores on a filamentous network of cells. (E) Gametophore buds are converted to rhizoid masses in plants transformed with both 35S:*PpRSL1* and 35S:*PpRSL2*.

(F) Young gametophore produces a mass of rhizoids in plants transformed with both 35S:*PpRSL1* and 35S:*PpRSL2*. (G) Older gametophore than that shown in (F).

(H) Fewer gametophores developed in plants transformed with both 35S:*PpRSL1* and 35S:*PpRSL2* than in wild type (D). (I and J) Transverse sections of wild-type gametophores showing the epidermal origin of basal and mid-stem rhizoids.

(K and L) Transverse sections of gametophores showing the epidermal origin of basal and mid-stem rhizoids in plants transformed with both 35S:*PpRSL1* and 35S:*PpRSL2*.

Scale bars = 250 µm in A, B, C, E, F and G; 1 mm in D and H; 100 µm in I, J, K, L.
Table 4.1 The number of gametophores in wild type and *PpRSL1*- and *PpRSL2*-overexpressing plants.

<table>
<thead>
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<th>Number of gametophores cm(^{-2}) (± standard deviation)</th>
<th>Student's t-test p value</th>
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<tr>
<td>Wild type</td>
<td>47.8 ± 9.16</td>
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<tr>
<td>35S:<em>PpRSL1</em></td>
<td>42.1 ± 5.74</td>
<td>0.34</td>
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<tr>
<td>35S:<em>PpRSL2</em></td>
<td>45.8 ± 6.97</td>
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<tr>
<td>35S:<em>PpRSL1</em>;35S:*PpRSL2</td>
<td>0.68 ± 0.79</td>
<td>0.0003</td>
</tr>
</tbody>
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Figure 4. 9 Images showing Pprsl1 Pprsl2 anatomy is identical to wild type
Transverse section through basal (A) and mid-stem (B) regions of a Pprsl1 Pprsl2 double mutant gametophore showing wild type cellular anatomy despite defectiveness of rhizoid development.
Scale bar = 100 µm
4.3.7 *PpRsl1*PpRsl2 double mutants are resistant to TIBA

If a polar auxin transport is involved in regulating rhizoid differentiation in *P. patens*, it would be expected that TIBA treatment would affect rhizoid differentiation in gametophores. This hypothesis was tested by investigating TIBA effect on rhizoid differentiation. TIBA treatment of wild type protonema suppressed rhizoid differentiation in gametophores (figure 4.10A, B). This suggests that polar auxin transport is involved in rhizoid differentiation in developing gametophores. Since class 1 *RSL* genes regulate rhizoid differentiation downstream of auxin, it was anticipated that mutant plants which lack both *PpRSL1* and *PpRSL2* functions would be resistant to TIBA. As expected, TIBA treatment did not have an effect on the morphology of the gametophores in *Pprsl1Pprsl2* double mutants (figure 4.10C, D). This also supports the proposal that class 1 *RSL* regulates rhizoid differentiation downstream of auxin.

4.4 Discussion

Rhizoids in non vascular plants, and root hairs in vascular plants share common roles in plant development; they help plants to anchor in substratum and to acquire nutrients (Peterson and Farquhar, 1996; Gahoonia et al., 1997; Duckett et al., 1998; Gahoonia and Nielsen, 1998). The developmental regulation of rhizoids and root hairs requires the function of class 1 *RSL* genes. Furthermore, the fact that the hairless phenotype of mutant plants which lack class 1 *AtRSL* is rescued by introducing class 1 *RSL* of *P. patens* suggests the function of class 1 *RSL* has been conserved. However, it is likely that the regulatory relationship between class 1 *RSL* and auxin has been changed during land plant evolution.
Figure 4. 10 Images showing TIBA treatment suppresses rhizoid development in developing gametophores.

A. Wild type gametophores grown on minimal media.
B. Wild type gametophores grown on 300µM TIBA-treated condition.
C. *Pprsl1 Pprsl2* gametophores grown on normal condition.
D. *Pprsl1 Pprsl2* gametophores grown on 300µM TIBA -treated condition.
Scale bar = 500 µm.
4.4.1 Mechanism of auxin action differs between rhizoid and root hair differentiation

Although auxin positively regulates root hair, and rhizoid development in *A. thaliana* and *P. patens*, this study suggests that the mechanism of auxin action differs between rhizoid and root hair differentiation. Root hair development in *A. thaliana* can be understood as having three distinct developmental steps, determination of cell identity, root hair initiation and elongation by tip growth, and each developmental step is controlled by specific transcription factors; *GL3, EG3, TTG, CPC* and *GL2* regulate cell fate determination while class 1 class 2 *RSL* genes control root hair initiation and elongation, respectively. During the root hair development, auxin regulates root hair initiation elongation while auxin is not involved in rooting cell fate determination. Recent studies with transgenic plants transformed with the *DR5promoter-GFP* construct showed that the intensity of GFP is stronger in atrichoblasts than in trichoblasts of *A. thaliana*. The difference of GFP intensity between trichoblasts and atrichoblasts doesn't exist in the mutant plants which lack the *AUX1* (Jones et al., 2009). Interestingly, *AUX1* is predominantly expressed in atrichoblasts but not expressed in trichoblasts. These results suggest that the endogenous concentration of auxin accumulated in atrichoblasts is higher than in trichoblasts. Because atrichoblasts of the wild type do not differentiate root hairs, these results also support that auxin accumulation is not sufficient for root hair differentiation from atrichoblasts, and auxin is not involved in determination of cell identity. These results are supported by the observations that auxin treatment of wild type *A. thaliana* does not increase root hair formations because auxin treatment does not cause ectopic root hair formation in atrichoblasts (Masucci and Schiefelbein, 1996).
In contrast to *A. thaliana*, no genes related to the determination of the rhizoid-forming cell fate have been reported in *P. patens*. Thus it is still unclear how they determine the rhizoid forming cell fate in developing gametophores. Instead, it is likely that auxin functions in the process that determines rhizoid forming cell fate in gametophores, at least in part. This study showed that auxin action in the rhizoid differentiation of *P. patens* differs from the auxin action in root hair differentiation of *A. thaliana*; plants transformed with *GmGH3* promoter:*GUS* show a strong expression of the *GUS* gene in the specialized epidermal cells which give rise to rhizoids compared with other types of cells, suggesting that a relatively high concentration of auxin is accumulated in rhizoid forming cells compared with other types of cells. Furthermore auxin treatment of wild-type *P. patens* increases the number of rhizoids formation (figure 4.4; Sakakibara et al., 2003). In protonema development, a gradient of auxin promotes the formation of a gradient of cell identity along a protonema. This suggests that, auxin acts as a morphogen – a signalling molecule whose cellular concentration determines cell fate – during protonema and gametophores development of *P. patens*.

4.4.2 Regulatory relationship between class 1 *RSL* and auxin differs between *Physcomitrella patens* and *Arabidopsis thaliana*

Auxin regulates rhizoid and caulonema differentiation by positively regulating class 1 *RSL* genes in *P. patens*; auxin treatment increases the transcriptional expression of class 1 *RSL*, and *PPrsl1 PPrsl2* double mutants are resistant to auxin. In *A. thaliana* class 1 *RSL* regulates root hair differentiation independently of auxin; auxin treatment of *AtRhd6 AtRsl1* double mutants induces root hair differentiation, and transcriptional expression levels of class 1 *RSL* genes in auxin treated conditions are almost the same to the auxin-untreated
controls (Yi et al., 2010). Instead, auxin positively regulates AtRSL4 expression which belongs to class 2 RSL and controls root hair elongation downstream of class 1 RSL. Together these data indicate that while auxin regulates RSL networks in P. patens and A. thaliana, its interaction with these networks is different in each species. If the moss RSL network represents the ancestral state that existed in early diverging groups of land plants, our data suggest that the way in which auxin controls the network changed during land plant evolution.

4.4.3 Class 1 RSL regulates rhizoids differentiate only from epidermal cells in P. patens.

The internal cellular anatomy of the Pprsl1 Pprsl2 mutant gametophores is identical to the wild type in spite of the obvious defects in rhizoid development. This indicates that PpRSL1 and PpRSL2 are required for the rhizoid differentiation but are not involved in the development of other types of cells such as cortex cells. These data are consistent with the observation in which co-overexpression of PpRSL1 and PpRSL2 do not develop rhizoid from cortex cells despite the massive rhizoid formation from epidermal cells compared to wild-type plants. These data suggest that epidermal cells develop special environments in which class 1 RSL genes activate rhizoid development. However, it is poorly understood what is special about epidermal cells in developing rhizoids compared to other types of cells.

In this study, I showed that auxin distribution or sensitivity are very different in epidermal cells and cortex cells; GmGH3promoter:GUS is highly expressed in epidermal cells compared to cortex cells. This suggests the possibility that auxin determines the different cell
environments inhabited by epidermal and cortex cells. However, it is unlikely to be true because previous studies of the auxin effect on rhizoid development showed that auxin treatment enhances rhizoid differentiation from the epidermis but not from cortex (Sakakibara et al., 2003). This indicates that auxin is not the totipotent factor to change cell identity of cortex cells into epidermal cells. Unfortunately, no mutant or overexpressing plants which develop rhizoids from non-rhizoid forming cells such as cortex and leaf cells was reported. This means that it is still difficult to answer to the question “what is special about epidermis”. More studies are required for expanding our understanding of the different cell identity and environment between epidermal and other types of cells in *P. patent*. 
Chapter Five:

General Discussion
This thesis describes class 1 RSL regulation and function in the caulonema and rhizoid differentiation of moss development. *P. patens* differentiates caulonema and rhizoids as specialized tip growing filamentous cells for nutrient acquisition and attachment to substratum. Although caulonema and rhizoids differentiate in different developmental stages, both caulonema and rhizoid differentiation are required for class 1 RSL regulation and function; *PpRSL1* and *PpRSL2* genes are predominantly expressed in the cells which develop into caulonema and rhizoids, and mutant plants which lack both *PpRSL1* and *PpRSL2* functions (*Pprsl1 Pprsl2*) strongly suppress caulonema differentiation in protonema and rhizoid differentiation in gametophores. Furthermore co-overexpression of *PpRSL1* and *PpRSL2* is sufficient to activate caulonema and rhizoid differentiation even in auxin-untreated conditions. These results indicate that class 1 RSL is necessary and sufficient for caulonema and rhizoid differentiation in *P. patens*.

Similarly, root hair differentiation in *A. thaliana* is positively regulated by class 1 RSL functions; *AtRHD6* and *AtRSL1* are expressed only in trichoblasts which differentiate root hairs, but not in root hair cells. Mutant plants which lack both *AtRHD6* and *AtRSL1* activity suppress root hair initiation, suggesting the hypothesis that the class 1 RSL as key regulators in rooting cell development has been conserved during land plant evolution. This hypothesis is indirectly supported by some phylogenetic analyses, showing many crucial genes controlling plant development have been conserved during land plant evolution (Nishiyama et al., 2003; Richardt et al., 2007). Furthermore, the fact the class 1 RSL genes exist in all land plants for which the genome sequence is available also supports the conserved role of the RSL gene in rooting cell development of land plants (Pires and Dolan, 2010).
Plants expanded their habitat from water to land and established the first continental vegetation 430 million years ago (Kenrick and Crane; 1997; Nishiyama et al., 2003). Subsequently, land plant evolution has resulted in the explosive increase of morphological diversity (Kenrick and Crane, 1997; Bateman et al., 1998; Richardt et al., 2007). It is hypothesized that modification of genes and rearrangement of gene regulatory networks give rise to the morphological diversity during evolution (Davison and Erwin; 2006; Peter and Davison; 2011) In this study, it was shown that despite the conserved function of RSL gene in rooting cell development, regulatory interaction between class 1 RSL and auxin is completely different between P. patens and A. thaliana; auxin regulates caulonema and rhizoid differentiation by positively regulating class 1 RSL genes in P. patens while auxin regulates root hair differentiation of A. thaliana independently of class 1 RSL. Furthermore, changed interaction between regulatory components in rooting cell development was also found in the study of class 2 RSL regulation in moss development. Class 1 RSL genes of P. patens do not regulate the transcriptional expression of class 2 RSL genes during moss development while class 1 RSL genes of A. thaliana regulate class 2 RSL gene expressions in root hair development; transcriptional expression levels of class 2 RSL genes between wild type and Pprsl1 Pprsl2 double mutant plants were almost identical (Pires, 2011), suggesting that interaction between class 1 and class 2 RSL genes has changed during land plant evolution, and rearrangement of regulatory systems results in morphological diversity of land plants.

In this study, I showed that PpRSL1 and PpRSL2 control caulonema and rhizoid development in P. patens. However, genes that regulate PpRSL1 and PpRSL2 expression have not been identified. Furthermore genes acting down stream of PpRSL1 and PpRSL2 have yet to be identified. In this respect, further studies of identifying downstream and
upstream components of *PpRSL1* and *PpRSL2* will be important to expand our understanding of filamentous rooting cell development and evolution. To identify downstream components of *PpRSL1* and *PpRSL2*, microarray and RNA-seq experiments will identify putative targets of PpRSL1 and PpRSL2 regulation. Characterization of the transcriptome of the *Pprsl1Pprsl2* mutant and *35S:PpRSL1;35S:PpRSL2* transgenic plants will be instructive. From the gene expression profiling data, we can identify the candidate genes as downstream components of *PpRSL1* and *PpRSL2*. If some components positively regulate filamentous rooting cell development downstream *PpRSL1* and *PpRSL2*, it is expected their expressions are decreased in *Pprls1Pprsl2* double mutant backgrounds, while increased in *35S:PpRSL1;35S:PpRSL2* transgenic plant backgrounds. Following characterization studies of the candidate genes using loss of function and gain of function approaches will help us understand downstream of class 1 *RSL* in caulonema and rhizoid development.

Ethyl methanesulfonate (EMS) mutagenesis and map-based cloning can be used for identifying upstream components of class 1 *RSL* in *P. patens*. If some genes are upstream regulators of class 1 *RSL*, and EMS treatment induces mutagenesis on the genes, it is expected that class 1 *RSL* expression level and pattern would be changed in the mutant plants. To improve the efficiency of mutant screening, EMS mutagenesis of transgenic marker lines such as *PpRSL1 promoter:GUS* or *PpRSL2 promoter:GUS* will be required. However, map-based cloning is almost impossible in *P. patens*, because of the lack of a genetic linkage map (Kamisugi et al., 2008). Furthermore, experimental crosses and identification of crossed sporophytes is also very difficult in *P. patens*. I think that the possible way for gene identification through EMS mutagenesis is whole genome sequencing using next generation sequencing technologies. The advanced sequencing
technology dramatically increases the amount of sequence output per run and improves the sequencing accuracy. The current next generation sequencing machines can read around 250 billion bases in a week (Xiong et al., 2011). Therefore a combination of EMS mutagenesis and next generation sequencing technology will allow us to identify upstream components of class 1 \textit{RSL} in \textit{P. patens}.

\textbf{References}


Grebe, M., Friml, J., Swarup, R., Ljung, K., Sandberg, G., Terlou, M., Palme, K.,

lower plant. Nature 259, 480-482.

protonema - comparison of its levels by protein-kinase and gilman assays. Plant
physiol. 59, 490-496.

component ARPC1 is required for proper cell morphogenesis and polarized cell
growth in Physcomitrella patens. Plant Cell 17, 2327-2339.

Development and Sexual-Differentiation in the Fern Ceratopteris-Thalictroides (L)

gene-trap and enhancer-trap systems in the moss Physcomitrella patens. Plant J 28,
105-116.

Hohe, A., Egener, T., Lucht, J.M., Holtorf, H., Reinhard, C., Schween, G., and Reski,
R. (2004). An improved and highly standardised transformation procedure allows
efficient production of single and multiple targeted gene-knockouts in a moss,

Jacobs, W.P. (1951). Studies on Cell-Differentiation - the Role of Auxin in Algae, with

rhizoid system development in early diverging land plants. Development, in press.

Jayaswal, R.K., and Johri, M.M. (1985). Occurrence and Biosynthesis of Auxin in
Protonema of the Moss Funaria-Hygrometrica. Phytochemistry 24, 1211-1214.

chloronemata of the moss &lt;i&gt;Physcomitrella patens:&lt;/i&gt; responses of


Appendix 1.

Southern blot analysis of 35S:PpRSL1;35S:PpRSL2 plants

A. Images of bud development in wild type (WT), and in two independent lines, transformed with both 35S:PpRSL1 and 35S:RSL2 (35S:PpRSL1;35S:RSL2–1 and 35S:PpRSL1 35S:RSL2 - 2). Scale bar = 100 µm

B. Southern blot analysis showing that each line transformed with 35S:PpRSL1 35S:PpRSL2 has more than two insertions. 35P-labeled NPT gene was used as a probe. On the left is a one day exposure, and on the right is a seven day exposure.
Appendix 2.

Genotyping of $PpRSL1_{promoter}:GUS$ and $PpRSL2_{promoter}:GUS$ plants

A. Each two individual lines of $PpRSL1_{promoter}:GUS$ and $PpRSL2_{promoter}:GUS$

B. Schematics showing $GUS$ insertion into $PpRSL1$ and $PpRSL2$ loci

C. PCR showing $GUS$ insertion into $PpRSL1$ and $PpRSL2$ loci. DNA bands on the gel were amplified with the each pair of $PpRSL1$ and $PpRSL2$ specific primer indicated in B (large arrows). Scale bar = 500 µm
### Appendix 3.

### Primers used in this study.

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<tr>
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</tr>
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</tr>
<tr>
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<tr>
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<tr>
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Appendix 4.

Publications


Auxin promotes the transition from chloronema to caulonema in moss protonema by positively regulating \( PpRSL1 \) and \( PpRSL2 \) in \( Physcomitrella patens \)

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Summary

- Protonemata are multicellular filamentous networks that develop following the germination of a haploid moss spore and comprise two different cell types – chloronema and caulonema. The ROOT HAIR DEFECTIVE SIX-LIKE1 (\( PpRSL1 \)) and \( PpRSL2 \) basic helix–loop–helix transcription factors and auxin promote the development of caulonema in \( Physcomitrella patens \) but the mechanism by which these regulators interact during development is unknown.
- We characterized the role of auxin in regulating the function of \( PpRSL1 \) and \( PpRSL2 \) in the chloronema-to-caulonema transition during protonema development.
- Here, we showed that a gradient of cell identity developed along protonemal filaments; cells were chloronemal in proximal regions near the site of spore germination becoming progressively more caulonemal distally as filaments elongated. Auxin controlled this transition by positively regulating the expression of \( PpRSL1 \) and \( PpRSL2 \) genes. Auxin did not induce caulonemal development in \( Pprsl1 \) \( Pprsl2 \) double mutants that lack \( PpRSL1 \) and \( PpRSL2 \) gene activity while constitutive co-expression of \( PpRSL1 \) and \( PpRSL2 \) in the absence of auxin was sufficient to program constitutive caulonema development.
- Together, these data indicate that auxin positively regulates \( PpRSL1 \) and \( PpRSL2 \) whose expression is sufficient to promote caulonema differentiation in moss protonema.

Introduction

The protonema is the first phase in the haploid stage of the life cycle of mosses such as \( Physcomitrella patens \). Protonemata are multicellular filamentous systems that develop when haploid spores germinate and comprise two filament types: chloronema and caulonema. The cells of chloronema contain large round chloroplasts and the cross-walls that form are oriented perpendicular to the long axis of the filament; the chloroplasts of caulonema cells are relatively elongated and the crosswalls are oblique (Duckett \( et al. \), 1998; Reski, 1998). The apical cells of both chloronema and caulonema extend by tip growth despite the different cytological organization of the growing regions in these cell types; the vacuole and chloroplasts may extend to the growing tip of chloronema while the growing tip of apical caulonema cells is always densely cytoplasmic (Duckett \( et al. \), 1998; Pressel \( et al. \), 2008). In addition to these ultrastructural differences, caulonema and chloronema grow at different rates (Cove & Knight, 1993; Duckett \( et al. \), 1998; Cove, 2000; Schween \( et al. \), 2003; Menand \( et al. \), 2007a). A new chloronema cell is produced every 24 h by the division of the distal-most cells at the tip of filaments, which elongate at rates of \( 6 \) \( \mu \)m h\(^{-1} \); caulonema cells are derived from cells at the tips of filaments which elongate at \( 20 \) \( \mu \)m h\(^{-1} \) and divide every 7 h.

The induction of caulonema differentiation during protonema development is positively regulated by auxin. Treatment of moss with auxin increases the number of filaments that develop as caulonema and the protonemata of mutants with defects in auxin signalling develop chloronema alone with little or no caulonema (Johri & Desai,
Furthermore, experimental overexpression of the positive regulator of auxin biosynthesis *PpSHI1* enhances caulonema differentiation (Eklund et al., 2010). The differentiation of caulonema cells also requires the activity of a pair of basic helix–loop–helix transcription factors called *Physcomitrella patens* ROOT HAIR DEFECTIVE SIX-LIKE (*PpRSL1* and *PpRSL2*). Double mutants that lack both *PpRSL1* and *PpRSL2* functions develop protonema consisting of chloronema (Menand et al., 2007b). It is unknown if *PpRSL* genes and auxin interact in the control of protonema development or if they independently control this process.

*RLS* genes control the development of root hairs in *Arabidopsis thaliana* and because auxin positively regulates the transcription of *AtRSL4* during root hair development (Yi et al., 2010), we set out to test the hypothesis that auxin acts upstream of *PpRSL1* and *PpRSL2* during the development of caulonema. We demonstrate that auxin positively regulates the gradual transition from chloronema to caulonema that occurs along protonemal filaments. Auxin promotes this transition by positively regulating the activity of *PpRSL1* and *PpRSL2*; mutants that lack both *PpRSL1* and *PpRSL2* activity do not develop caulonema even when treated with auxin. Furthermore, constitutive coexpression of *PpRSL1* and *PpRSL2* in the absence of auxin is sufficient to activate caulonema differentiation during protonemal development. Together, these data indicate how auxin and *PpRSL1* and *PpRSL2* interact to control the gradual transition of cell fates along a developmental gradient.

**Materials and Methods**

Plant materials, growth and supplements

The Gransden wild-type strain of *P. patens* (Hedw.) Bruch and Schimp was used in this study (Ashton et al., 1979). Plants were grown at 25°C in a light regime of 16 h light:8 h dark with a quantum irradiance of 40 µmol m−2 s−1. For the growth of protonema, spores were inoculated on a 9 cm Petri dish containing solid minimal media overlaid with a cellophane disk (AA packaging, Preston, UK) for 1 or 2 wk (Ashton et al., 1979). *Pprsl1 Pprsl2* double mutants, *35S:PpRSL1;35S:PpRSL2* and *PpRSL1promoter::GUS* and *PpRSL2promoter::GUS* lines were described previously (Menand et al., 2007b; Jang et al., 2011). Auxin treatment of *P. patens* was carried out by plating spores in solid minimal media supplemented with 1 µM α-naphthalene acetic acid (Sigma) and incubating for 2 wk.

Reverse-transcription polymerase chain reaction (RT-PCR analysis)

Total RNA from 2-wk-old protonemal tissues were extracted with the RNeasy Plant Mini Kit (Qiagen). One microgram of total RNA was used for cDNA synthesis with the Superscript III reverse transcriptase (Invitrogen) and oligo dT primers. Polymerase chain reaction was carried out with equivalent amounts of cDNA template for amplification of fragments of *PpRSL1* (EF156393), *PpRSL2* (EF156394), and GAPDH (X72381).

**GUS staining**

One-week old and 2-wk-old protonema transformed with either the *PpRSL1promoter::GUS* or the *PpRSL2promoter::GUS* constructs were grown in standard growth conditions (already described) and incubated in glucuronidase (GUS) staining solution (100 mM NaPO₄ (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-glucuronide and 0.2% Triton X-100) at 37°C for 2–24 h. The samples were then washed with 100 mM NaPO₄ (pH 7.0) and incubated in 70% ethanol at 4°C for 3 d.

**Phenotypic analysis**

Spores for *Pprsl1 Pprsl2* and protoplasts of *35S:PpRSL1;35S:PpRSL2* were germinated on solid minimal media overlaid with cellophane disks and grown for the indicated number of days. Images were captured with a Pixera Pro ES600 camera mounted on Nikon Eclipse 800 and measured using Photoshop 7.0 (Adobe, Uxbridge, UK). Cell cycle times were determined by calculating rates of cell production. Cell production rates were calculated by dividing the time interval (in hours) during which colonies were growing by the number of cells produced in protonemal filaments during that time interval. Cell numbers were determined by counting the numbers of crosswalls that formed in a filament.

**Results**

There is a gradual transition from chloronema to caulonema in moss protonema

*P. patens* spores germinate and develop filamentous systems of protonema that consist of two cell types, chloronema and caulonema (Fig. 1). Caulonema divide transversely where the newly formed wall is perpendicular to the sidewalls of the cell; caulonema are longer and thinner than chloronema and new cell walls are oriented obliquely across the cell (Reski, 1998). Germinating spores produce two (2.17 ± 0.39 (SD) n = 12 germinating spores) branching protonemal filaments (Fig. 1a). The first cells to differentiate in these filaments have transverse crosswalls and characteristic morphology of chloronema (Fig. 1a,c; Cove & Knight, 1993). By 7 d after germination cell number in filaments increased to 9 or 10 cells (9.6 ± 0.52 (SD) n = 10 filaments) as a result of cell division in the apical cell.
Distal cells in these 7-d-old filaments are longer and narrower than the chloronema cells in the proximal region (Fig. 1d–f); the first cells that differentiated along the filament were $48.9 \pm 5.25\, \mu m$ (SD) long, the fifth cells were $66.2 \pm 7.49\, \mu m$ (SD) long and the sub-apical cells were $96.7 \pm 12.03\, \mu m$ (SD) long ($n \geq 13$ filaments). Furthermore, the distal cells in the 7-d-old filament develop oblique crosswalls characteristic of caulonema cells (Fig. 1d). This suggests that there is a transition from chloronema to caulonema within the first 7 d of protone mal development; the first proximal cells in filaments differentiate as chloronema cells while distal cells become progressively more caulonemal in character as development proceeds.

To confirm independently that there is a gradual transition from chloronema to caulonema along a protonemal filament we characterized the cell cycle times of cells in the early stages of protonemal development. The cell cycle of chloronema and caulonema is c. 24 and 7 h, respectively (Cove & Knight, 1993; Schween et al., 2003). The gradual transition from chloronema to caulonema suggests the hypothesis that cell cycle times would decrease along the length of a filament. To test this hypothesis we determined the cell cycle times of cells along wild-type protonemal filaments. The cell cycle of the first three cells that developed between germination and day 3 in the filament was c. 24 h and this is consistent with our conclusion that these cells are chloronema (Table 1). Cell cycle times progressively decreased in apical cells that formed later in development after day 3; from 18.9 h at 4 d to 12.1 h at 10 d (Table 1). These data indicate that the cell cycle times of the first three cells of the filament are characteristic of chloronema. Cell cycle times then progressively decrease as cells undergo the gradual transition to caulonema. Together, these data indicate that there is a gradual transition from chloronema to caulonema cell identity along a protonemal filament.

**PpRSL1 and PpRSL2 are required for the transition from chloronema to caulonema**

*PpRSL1* and *PpRSL2* encode basic helix–loop–helix transcription factors that are required for caulonema differentiation (Fig. 2m,n; Menand et al., 2007b). It is hypothesized that *Pprsl1 Pprsl2* double mutants only develop chloronema because chloronema is the default differentiation state in the absence of *PpRSL1* and *PpRSL2* activity. If cells of *Pprsl1 Pprsl2* protonema are transformed...
into chloronema we would expect that the gradient in cell length that develops in the wild type would not develop along filaments in these double mutants. Conversely, if chloronema cells were partially converted to caulonema in the double mutant, we would expect a partial gradient to form. To test this hypothesis we characterized the cell length gradient in Pprsl1 Pprsl2 double mutants (Fig. 2). The gradient of cell lengths that is characteristic of wild-type protonema development does not form in the Pprsl1 Pprsl2 double mutant (Fig. 2o). Instead, Pprsl1 Pprsl2 double

<table>
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<th>0–3 d</th>
<th>3–4 d</th>
<th>3–6 d</th>
<th>3–8 d</th>
<th>3–10 d</th>
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<tr>
<td>WT</td>
<td>24.4 ± 4.60 h</td>
<td>18.9 ± 6.41 h</td>
<td>15.9 ± 1.95 h</td>
<td>13.1 ± 1.56 h</td>
<td>12.1 ± 0.6 h</td>
</tr>
<tr>
<td>Pprsl1Pprsl2</td>
<td>24.0 ± 3.79 h</td>
<td>24.0 ± 0 h</td>
<td>24.8 ± 5.01 h</td>
<td>25.1 ± 3.63 h</td>
<td>24.4 ± 3.25 h</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
mutant cell lengths remain at c. 50 μm (51.9 ± 4.67 μm (SD) n = 82 filaments) along the entire protonemal filament while cell lengths progress from c. 50 μm to > 70 μm in the wild type by the time the sixth cell of the protonema filament has differentiated (Fig. 2a). Cells in the Pprsl1 Pprsl2 double mutant are therefore chloronemal in identity, resembling the first cells that differentiate along a wild-type protonemal filament. These data indicate that PpRL1 and PpRL2 activities are required for development of the gradient cell identity that occurs along wild-type protonemal filaments.

If all cells in the Pprsl1 Pprsl2 double mutant are chloronemal we would expect the cell cycle to be c. 24 h in all cells along the filament with no distal decrease characteristic of the wild type. Indeed, cell cycle times remained unchanged, at c. 24 h throughout the development of mutant protonemal filament (Table 1); 3, 4, 6, 8 and 10-d-old protonemal filaments of Pprsl1 Pprsl2 contained 3 ± 0.56 (SD), 3.91 ± 0.49 (SD), 5.57 ± 0.49 (SD), 7.87 ± 0.60 (SD) and 10 ± 0.86 (SD) (n ≥ 12 filaments) cells, respectively (Fig. 2p). By contrast, cell cycle times of the apical cells in wild-type protonemal filaments gradually decreased (Table 1). Because of the shorter cell cycle time there were more cells in the wild type (16.9 ± 0.69 (SD) n = 13 filaments) filament than in the Pprsl1 Pprsl2 double mutant (10.0 ± 0.86 (SD) n = 16 filaments) in 10-d-old plants (Fig. 2p). Together, these data indicate that PpRL1 and PpRL2 are required for the transition from chloronema to caulonema along protonemal filaments.

PpRL1 and PpRL2 genes are expressed in protonema

The demonstration that PpRL1 and PpRL2 control the transition from chloronema to caulonema leads to the hypothesis that these genes would be expressed in developing protonema. The RT-PCR revealed that PpRL1 and PpRL2 transcripts accumulated in protonema. Furthermore, steady-state levels of PpRL1 and PpRL2 mRNA were identical to wild type in Pprsl2 and Pprsl1 single mutants respectively (Fig. 3a). This indicates that PpRL1 activity is not required for transcriptional regulation of PpRL2, and PpRL2 is not required for transcriptional regulation of PpRL1. To image the spatial pattern of PpRL1 and PpRL2 transcription we generated plants transformed with PpRL1promoter:GUS and PpRL2promoter:GUS fusions. The expression of PpRL1promoter:GUS shows that PpRL1 is transcribed in a gradient; PpRL1 expresses strongly in proximal region of filaments at the centre of colonies, but the gene is not expressed in the distal ends of filaments at the edges of colonies (Fig. 3c–d). PpRL2 is most strongly expressed in the proximal ends of filaments at centre of the colony but is also expressed in cells at the colony edge (Fig. 3e–g). This suggests that while there may be a gradient of PpRL2 expression it is much less than PpRL1. Together, these data indicate that PpRL1 and PpRL2 are expressed in protonema and PpRL1 is expressed most highly in chloronema cells which develop into caulonema.

![Fig. 3](image-url)
Auxin positively regulates PpRSL1 and PpRSL2 expression

As both auxin and PpRSL genes positively regulate the transition from chloronema to caulonema, we tested the hypothesis that auxin controls this transition by positively regulating PpRSL1 and PpRSL2 activity. To determine if steady-state levels of PpRSL1 and PpRSL2 were regulated by auxin we carried out RT-PCR using mRNA that was isolated from wild-type cultures that were treated with the synthetic auxin, α-naphthalene acetic acid (NAA). A 20-h treatment with 10 μM NAA and a 14-d treatment with 1 μM NAA increased steady-state levels of PpRSL1 and PpRSL2 mRNA compared with untreated controls (Fig. 4a). This indicates that auxin positively regulates the transcription of both PpRSL1 and PpRSL2.

To determine if auxin regulates the spatial pattern of PpRSL1 and PpRSL2 gene expression we determined the effect of NAA treatment on the activity of PpRSL1promoter::GUS and PpRSL2promoter::GUS reporter. The NAA treatment increased the expression of GUS compared with untreated controls. Importantly, the expression of PpRSL1promoter::GUS extended out to the tips of the filaments in NAA-treated plants, while expression was undetectable in these distal regions in the untreated controls. Therefore, while the level of PpRSL1 expression was increased, NAA treatment also changed the spatial pattern of PpRSL1promoter::GUS expression (Fig. 4b,c). Auxin treatment also increased PpRSL2promoter::GUS expression (Fig. 4d,e). Nevertheless, because PpRSL2 is expressed throughout the protonema, auxin treatment did not change the spatial pattern of expression.

Auxin-induced caulonema differentiation requires PpRSL1 and PpRSL2 gene activity

If auxin positively regulates PpRSL1 and PpRSL2 activity during protonema development we predicted that Pprsl1 Pprsl2 double mutants would be resistant to auxin treatment. The NAA treatment of wild-type colonies resulted in the development of extensive growth of caulonema compared with untreated controls. By contrast, no caulonema developed in Pprsl1 Pprsl2 double mutants after auxin treatment. That is, auxin-treated and untreated Pprsl1 Pprsl2 double mutants were morphologically identical and therefore, the Pprsl1 Pprsl2 double mutant is resistant to the effects of auxin treatment (Fig. 5). This is consistent with the hypothesis that auxin controls the development of caulonema by positively regulating PpRSL1 and PpRSL2 activity.

PpRSL1 and PpRSL2 gene expression is sufficient for caulonema differentiation from chloronema

As auxin promotes caulonema development by positively regulating the expression of both PpRSL1 and PpRSL2, we predicted that plants that co-overexpress PpRSL1 and PpRSL2 would develop phenotypes resembling wild-type plants grown in the presence of auxin. As predicted the development of chloronema is repressed in plants expressing both PpRSL1 and PpRSL2 under the control of the 35S promoter. Instead, these 35S:PpRSL1;35S:PpRSL2-transformed colonies were composed almost entirely of caulonema and no gradient in cell length developed (Fig. 6). These data support the hypothesis that auxin promotes caulonema development by positively regulating PpRSL1 and PpRSL2 activity and demonstrates that coexpression of PpRSL1 and PpRSL2 is sufficient for the development of caulonema.

Discussion

The first cells to differentiate during protonema development are chloronemal and then after c. 4 d each new cell
that is produced at the growing tip of the filament is progressively more caulonemal in character. We showed here that *PpRSL1* and *PpRSL2* are required for the development of this chloronema–caulonema gradient and expression of *PpRSL1* progressively decreases as cells become more caulonemal in character. Consequently, *PpRSL1* is strongly expressed in cells that give rise to caulonema but not expressed in the mature caulonema itself, while intermediate levels of *PpRSL1* mRNA accumulate in cells undergoing the chloronema-to-caulonema transition. This suggests that the gradient in cell identity that forms during the chloronema-to-caulonema transition is likely generated by a gradient in *PpRSL1* expression. The demonstration that *PpRSL1* is most strongly expressed in cells that give rise to caulonema but not in caulonema itself is similar to *A. thaliana* where *AtRHD6* is expressed in the cells (trichoblasts) that give rise to root hair cells but not in the root hair cell itself. The principal difference between these two developing systems is that the gradient of *PpRSL1* expression is accompanied by a gradual transition between chloronema to caulonema in *P. patens* while the abrupt transition from trichoblast to root hair cell is controlled by a sudden downregulation of *AtRHD6* expression in *A. thaliana*. We predict that, together, *PpRSL1* and *PpRSL2* genes positively regulate the expression of genes that are required specifically for the differentiation and growth of caulonema cells in the same way that *AtRHD6* and *AtRSL1* genes control the expression of genes that control differentiation and growth of root hair cells *A. thaliana*.

**Fig. 5** *Pprsl1Pprsl2* protonema are resistant to auxin. (a) Physcomitrella patens wild-type protonema in control conditions. (b) Distal region of wild-type protonemal filaments in control conditions. (c) Wild-type protonema treated with auxin. (d) Distal region of wild-type protonemal filaments treated with auxin. (e) *Pprsl1 Pprsl2* double mutant protonema in control conditions. (f) Distal region of *Pprsl1 Pprsl2* double mutant protonemal filaments in control conditions. (g) *Pprsl1 Pprsl2* double mutant protonema treated with auxin. (h) Distal region of *Pprsl1 Pprsl2* double mutant protonemal filaments treated with auxin. Bars: (a,c,e,g) 500 μm, (b,d,f,h) 100 μm.

*RSL* genes control the development of caulonema and rhizoids in *P. patens* and root hairs in *A. thaliana* (Masucci & Schiefelbein, 1994; Menand *et al.*, 2007b). While each of these cell types elongate by tip growth, not all tip growing cells are regulated by *RSL* genes. For example the cells of moss chloronema extend by tip growth but *RSL* genes do not control their development (Menand *et al.*, 2007a,b). Similarly the growth and development of tip-growing pollen tubes is independent of *RSL* gene function in *A. thaliana* (Menand *et al.*, 2007b). This indicates that the *RSL* genes are not regulators of all tip-growing cells. In other words, *RSL* genes are not simply positive regulators of tip growth. Instead, *RSL* genes control the development of tip-growing cells that form at the interface of the plant and its substratum and are likely to have roles in nutrient uptake and anchorage. It has been hypothesized that caulonema are involved in nutrient uptake and transport during protonemal growth and it is likely that rhizoids may play a similar function in nutrient uptake as well as playing a role in anchorage (Duckett *et al.*, 1998). Similarly root hairs are important for nutrient uptake and anchorage in angiosperms (Peterson & Farquhar, 1996; Gahoonia *et al.*, 1997; Gahoonia & Nielsen, 1998). Therefore, it is possible that *RSL* genes control the development of a subset of tip-growing cells: tip-growing cells that are active in nutrient uptake, anchorage and develop at the interface of the plant and its substratum.

Auxin positively regulates the chloronema-to-caulonema transition in *P. patens* by positively regulating *PpRSL1* and
PpRSL2 expression. Auxin also controls the development of root hairs in *A. thaliana* but it does not regulate the transcription of *AtRHD6* and *AtRSL1* – the *A. thaliana* genes that are most similar to *PpRSL1* and *PpRSL2*. This indicates that the mechanism of auxin-regulated development is different in *P. patens* caulonema and *A. thaliana* root hairs. In the latter case, auxin controls root hair development by regulating the expression of a different group of *RSL* genes: auxin positively regulates the expression of *AtRSL4* and negatively regulates *AtRSL2* (Yi et al., 2010). Therefore, despite the differences in the precise nature of the interaction between *RSL* genes and auxin, the regulation of *RSL* genes by auxin is ancient and was present in the last common ancestor of the angiosperms and the mosses. Nevertheless, *RSL* genes have not yet been identified in the genomes of extant members of the earliest diverging group of land
plants, the liverworts or in their sister group, the streptophyte algae. It is possible that RSL genes are present in these taxa but have not yet been identified because there are no complete genome sequences available for these organisms. Nevertheless, no RSL genes were found in the complete genome sequences of *Chlamydomonas reinhardtii* or *Ostreococcus tauri*. This suggests that RSL genes evolved sometime after the divergence of the chlorophytes from the streptophytes but before the evolution of the mosses (Pires & Dolan, 2010). In other words, the RSL pathway either originated among the streptophyte algae or among the earliest land plants. Identification of RSL genes among liverworts and the streptophyte algae and their functional characterization will define when this regulatory module first evolved.

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**References**


RSL genes are sufficient for rhizoid system development in early diverging land plants

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SUMMARY

Land plants are anchored to their substratum from which essential inorganic nutrients are taken up. These functions are carried out by a system of rhizoids in early diverging groups of land plants, such as mosses, liverworts and hornworts. *Physcomitrella patens* RHD SIX-LIKE1 (PpRSL1) and PpRSL2 transcription factors are necessary for rhizoid development in mosses. Similar proteins, AtRHD6 and AtRSL1, control the development of root hairs in Arabidopsis thaliana. Auxin positively regulates root hair development independently of AtRHD6 and AtRSL1 in *A. thaliana* but the regulatory interactions between auxin and PpRSL1 and PpRSL2 are unknown. We show here that co-expression of PpRSL1 and PpRSL2 is sufficient for the development of the rhizoid system in the moss *P. patens*; constitutive expression of PpRSL1 and PpRSL2 converts developing leafy shoot axes (gametophores) into rhizoids. During wild-type development, PpRSL1 and PpRSL2 are expressed in the specialized cells that develop rhizoids, indicating that cell-specific expression of PpRSL1 and PpRSL2 is sufficient to promote rhizoid differentiation during wild-type *P. patens* development. In contrast to *A. thaliana*, auxin promotes rhizoid development by positively regulating PpRSL1 and PpRSL2 activity in *P. patens*. This indicates that even though the same genes control the development of root hairs and rhizoids, the regulation of this transcriptional network by auxin is different in these two species. This suggests that auxin might have controlled the development of the first land plant soil anchoring systems that evolved 465 million years ago by regulating the expression of RSL genes and that this regulatory network has changed since mosses and angiosperms last shared a common ancestor.

KEY WORDS: Basic helix-loop-helix transcription factor, Rhizoid, Root hair, Root system evolution, Physcomitrella, Arabidopsis

INTRODUCTION

The development of specialized tip-growing filamentous rhizoids in early diverging groups of land plants was crucial for the establishment of the first continental vegetation sometime before 465 million years ago (Kenrick and Crane, 1997; Bateman et al., 1998; Wellman and Gray, 2000; Gensel and Edwards, 2001; Wellman et al., 2003; Raven and Crane, 2007). The algal ancestors of the land plants lived in water and absorbed nutrients across their entire surface and some developed specialized rhizoids that functioned to anchor these plants in sediments from which nutrients were extracted (Box et al., 1984; Box, 1986; Box, 1987; Karol et al., 2001; Raven and Edwards, 2001). Once plants moved onto land, they developed photosynthetic organs that grew into the air where inorganic mineral nutrients were not available (Niklas, 1997). Systems of filamentous rhizoids and roots with associated filamentous root hairs anchored plants in place and supported growth in height (Raven and Edwards, 2001). The entire anchorage system of these early diverging groups of non-vascular land plants comprised tip-growing filamentous rhizoid cells (Kenrick and Crane, 1997; Bower, 1929). In mosses, rhizoids are multicellular filamentous cells. Two populations of rhizoids in Physcomitrella patens can be distinguished by the position on gametophores: basal rhizoids form in basal regions of the gametophore whereas mid-stem rhizoids develop in more apical regions (Sakakibara et al., 2003). The basal rhizoids differentiate from any epidermal cell near the base of the young gametophores whereas mid-stem rhizoids differentiate from the epidermal cells, which are located to the outside of two small leaf trace cells just below adult leaves.

Auxin positively regulates the development of both basal and mid-stem rhizoids in *P. patens*; treatment of wild-type plants with auxin increases the number of rhizoids that develop (Ashton et al., 1979; Sakakibara et al., 2003). Therefore, it is likely that auxin positively regulates the expression of genes required for rhizoid development. One such gene is *PpHb7*, which encodes an HD-Zip I subfamily protein required for late rhizoid differentiation; mutants that lack *PpHb7* function develop rhizoids that are indistinguishable from wild type except that they fail to form the red-brown pigment that normally accumulates late in the differentiation of wild-type rhizoids (Sakakibara et al., 2003). PpRSL1 and PpRSL2 are basic-helix-loop-helix (bHLH) transcription factors that are required for the development of rhizoids; mutants that lack *PpRSL1* and *PpRSL2* activity develop few very short basal rhizoids, indicating that *PpRSL1* and *PpRSL2* act early in rhizoid development (Menand et al., 2007). Nevertheless, the regulatory relationship between *PpRSL1* and *PpRSL2* and auxin has not been elucidated.

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As *PpRSL1* and *PpRSL2* are necessary for rhizoid development in *P. patens*, we set out to determine whether the expression of both *PpRSL* genes was sufficient for rhizoid development. We show here that co-expression of *PpRSL1* and *PpRSL2* is sufficient to transform gametophore cells into rhizoid cells, indicating that *PpRSL1* and *PpRSL2* expression is sufficient for rhizoid system development. We also show that auxin positively regulates rhizoid development by promoting cell-specific *PpRSL1* and *PpRSL2* transcription. Together these data suggest that auxin-induced expression of RSL genes controlled the development of early land plant rhizoids systems.

**MATERIALS AND METHODS**

**Plant materials, growth and supplements**

The Gransden wild-type strain of *Physcomitrella patens* (Hedw.) Bruch and Schimp was used in this study (Ashton et al., 1979). Cultures were grown at 25°C and illuminated with a light regime of 16/8 hours (light/dark) and a quantum irradiance of 40 μE m−2 s−1. For the growth of gametophores, spores were inoculated on a 9 cm Petri dish containing solid minimal media overlaid with a cellophane disk (AA packaging) for 3 weeks (Ashton et al., 1979). Protonemal samples for transformation were grown on minimal media supplemented with 5 mg/l NH4 and 50 mg/l glucose for 1 week. *P. patens* *PpRsl1*, *Pprs2* and *Pprsl2* double mutants, as well as *A. thaliana* *AtRSL1* promoter:GFP:*AtRSL1* and *AtRHD6* promoter:GFP:*AtRHD6* lines were previously described (Menand et al., 2007). For the phenotypic analysis of plants, small protonemal inocula were placed on solid minimal media overlaid with cellophane disks and grown for 3 weeks. Auxin treatment of *P. patens* was carried out by transferring 3-week-old plants to solid minimal media supplemented with 1 μM α-naphthalene acetic acid (Sigma) and incubating for 1 week.

**Construction of constitutively overexpressing vector**

For constitutive overexpression of both *PpRSL1* and *PpRSL2* at the same time, the 35S:*PpRSL1*;35S:*PpRSL2* vector was generated. To obtain 35S:*PpRSL1*:NOStet DNA cassette including *SpeI* and *ApaI* enzymatic restriction sites, two different vectors were used: pCAMBIA-35S:*PpRSL1* carrying 35S:*PpRSL1*:NOStet cassette (Menand et al., 2007) and pBluescript II SK + (Stratagene) having *SpeI* and *ApaI* sites. First, pCAMBIA-35S:*PpRSL1* was excised with *EcoRI* and *HindIII* to obtain 35S:*PpRSL1*:NOStet cassette and this cassette was inserted into the pBluescript vector which had been digested with *EcoRI* and *HindIII*. pBluescript-35S:*PpRSL1* carrying SpeI-35S:*PpRSL1*:NOStet-*ApaI* cassette was obtained by ligation. The SpeI-35S:*PpRSL1*:NOStet-*ApaI* cassette, which was obtained by digesting pBluescript-35S:*PpRSL1* with *SpeI* and *ApaI*, was inserted into pPpRSL1-KO vector carrying partial genomic DNA fragments of *PpRSL1* for homologous recombination (Menand et al., 2007). The vector was named 35S:*PpRSL1*. To obtain 35S:*PpRSL2*:NOStet cassette carrying *NorI* and *AupI* sites two different vectors, pCAMBIA-35S:*PpRSL2* carrying 35S:*PpRSL2*:NOStet cassette and pGEM-T Easy (Promega) including *NorI* and *AupI* sites were used. pCAMBIA-35S:*PpRSL2* was obtained by amplifying the *PpRSL2* coding sequence from protonema cDNA with 35S:*PpRSL2* primers and cloning it into the BamH1 and SalI sites of pCAMBIA 1300 (Menand et al., 2007). 35S:*PpRSL2*:NOStet cassette was obtained by digesting pCAMBIA-35S:*PpRSL2* with *EcoRI* and *SpeI*. This cassette was inserted into pGEM-

![Fig. 1. *PpRSL1* and *PpRSL2* gene expression is sufficient for rhizoid system development in *P. patens*. A-D, I and J show wild-type plants; E-H, K and L show plants transformed with 35S:*PpRSL1* 35S:*PpRSL2*. (A) Wild-type gametophore bud that has just started to develop rhizoids but not yet begun to form leaves. (B) Young wild-type gametophore with the first juvenile leaves. (C) Older gametophore than that shown in B. (D) Three-week-old wild-type protonema with developing leafy gametophores on a filamentous network of cells (protonema). (E) Gametophore buds are converted to rhizoid masses in plants transformed with both 35S:*PpRSL1* and 35S:*PpRSL2*. (F) One of the rare gametophores that form on protonema transformed with 35S:*PpRSL1* and 35S:*PpRSL2*. This gametophore produces a mass of rhizoids and is the same age as the wild-type gametophore shown in B. (G) Older gametophore than that shown in F. Large numbers of rhizoids develop on the gametophores transformed with both 35S:*PpRSL1* and 35S:*PpRSL2* compared with wild-type gametophores of the same size and age. (H) Fewer gametophores developed in plants transformed with both 35S:*PpRSL1* and 35S:*PpRSL2* than in wild type (D). (I) Transverse section of wild-type gametophores showing the epidermal origin of basal rhizoids. (J) Transverse section of wild-type gametophores showing the epidermal origin of mid-stem rhizoids. (K) Transverse section showing the epidermal origin of mid-stem rhizoids in the gametophores of plants transformed with both 35S:*PpRSL1* and 35S:*PpRSL2*. Scale bars: 250 μm in A-C,E-G; 1 mm in D,H; 100 μm in I-L.
PpRSL1 promoter region was inserted into AscI and ApaI sites of pBHSNR-GUS and another fragment directly downstream of dIII sites. HinII selected on Hygromycin B (25 μg/ml). For the transformations of 35S:PpRSL1 and 35S:PpRSL2 vectors, genomic DNA fragments upstream of PpRSL1 gene were amplified by PCR. The genomic DNA fragment upstream of PpRSL1 promoter was inserted into AscI and ClaI sites of pBHSNR-GUS and another fragment directly downstream of PpRSL1 promoter region was inserted into BamHI and HindIII sites of pBHSNR-GUS. For the PpRSL2 promoter:GUS vector construction two genomic DNA fragments of PpRSL2 gene were amplified by PCR. The genomic DNA fragment upstream of PpRSL2 promoter was inserted into AscI and ClaI sites of pBHSNR-GUS and another fragment directly downstream of PpRSL2 promoter was inserted into SmaI and HindIII sites.

**P. patens transformation**

PEG transformation of *P. patens* protoplasts was carried out as described previously (Schaefer and Zryd, 1997). The 35S:PpRSL1:35S:PpRSL2 vector and 35S:PpRSL1 vector were linearized with *AvrII* and *SwaI* before protoplast transformation whereas 35S:PpRSL2 vector was linearized with *SwaI*. Transformants for 35S:PpRSL1;35S:PpRSL2 and 35S:PpRSL1 were selected on G418 (50 μg/ml) and transformants for 35S:PpRSL2 were selected on Hygromycin B (25 μg/ml). For the transformations of PpRSL1 promoter:GUS and PpRSL2 promoter:GUS vectors was linearized with *AscI* and *HindIII* before protoplast transformation whereas the PpRSL2 promoter:GUS vector was linearized with *AscI* and *BamHI*. Transformants were selected on Hygromycin B (25 μg/ml).

**RT-PCR analysis**

Total RNA from 4-week-old gametophores was extracted with the RNeasy Plant Mini Kit (Qiagen). Total RNA from 4-week-old gametophores was extracted with the RNeasy Plant Mini Kit (Qiagen). One microgram of total RNA was used for cDNA synthesis with the SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primers. PCR was carried out with equivalent amounts of cDNA template for amplification of fragments of PpRSL1 and PpRSL2 and GAPDH (X72381) (see Table S1 in the supplementary material).

| Table 1. The number of gametophores in wild type and PpRSL1- and PpRSL2-overexpressing plants |
|-------------------------------------------|-----------------------------------------------|
| **Number of gametophores cm⁻² (± s.d.)**   | **P-value (Student’s t-test)**                   |
| Wild type                                 | 47.8±9.16                                      |
| 35S:PpRSL1                                 | 42.1±5.74                                      |
| 35S:PpRSL2                                 | 45.8±6.97                                      |
| 35S:PpRSL1;35S:PpRSL2                      | 0.68±0.79                                      |

GUS staining analysis, embedding and sectioning

Isolated gametophores were incubated in GUS staining solution [100 mM NaPO₄ (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-glucuronide, 1 mM potassium ferricyanide and 0.2% Triton X-100] at 37°C for 2-14 hrs. The samples were then washed with 100 mM NaPO₄ (pH 7.0) and incubated in 70% EtOH. Whole-mounted samples were imaged with a Nikon Coolpix 995 camera mounted on a Leica Wild M10 stereomicroscope. For transverse sectioning, gametophores were aligned on a 1 mm layer of solid 1% agarose and covered with 1% molten agarose. After solidification, samples were cut into small blocks and washed twice in double distilled water for 15 minutes, then dehydrated in a graded series of ethanol (25, 50, 75 and 100%), for 30 minutes each. The dehydrated samples were sequentially incubated in a series of Technovit 7100 cold-polymerizing resin [33, 66 and 100% (v/v) in EtOH], for one hour each. Samples were then incubated in 100% Technovit for one day and placed in a plastic moulds. To solidify samples, a 15:1 (v/v) mixture of Technovit and hardener solution II was treated at room temperature for one day. Sections (10 μm) were taken from gametophores with an Ultracut E (Reichert-Jung). Images were captured with a Pixera Pro ES600 camera mounted on Nikon Eclipse 800.

**Phylogenetic analysis**

The bHLH domain of the RSL proteins were used for the phylogenetic analyses: AtRHD6 (At1g66470), AtRSL1 (At5g37800), AtRSL2 (At4g33880), AtRSL3 (At2g14760), AtRSL4 (At1g27740) and AtRSL5 (At5g3175), PpRSL1 (EF156393), PpRSL2 (EF156394), PpRSL3 (EF156395), PpRSL4 (EF156396), PpRSL5 (EF156397) PpRSL6 (EF156398), PpRSL7 (EF156399). AtIND (At4g00120) was used as an outgroup. The Bayesian analysis was performed with MrBayes version 3.1.2 (http://mrbayes.csit.fsu.edu/): two independent runs were computed for 240,000 generations, at which point the standard deviation of split frequencies was less than 0.01; one tree was saved every 100 generations, and 1800 trees from each run were summarized to give rise to the final cladogram. Trees were visualized using the program Figtree (http://tree.bio.ed.ac.uk/software/figtree/).
RESULTS

**PpRSL1 and PpRSL2 expression is sufficient for rhizoid system development in *P. patens***

Given the key function of RSL genes in rhizoid and root hair development in diverse groups of land plants, we tested the hypothesis that *PpRSL1* and *PpRSL2* are master regulators of rhizoid development in *P. patens* (Menand et al., 2007). To determine whether the activity of either *PpRSL1* or *PpRSL2* was sufficient to promote rhizoid development in *P. patens*, we expressed each gene individually at high levels using the CaMV35S promoter (Benfey and Chua, 1990). Plants were transformed with either 35S:*PpRSL1* or 35S:*PpRSL2* constructs. RT-PCR analysis demonstrated that steady state levels of *PpRSL1* and *PpRSL2* transcript was elevated in each transformed line, respectively (see Fig. S1 in the supplementary material).

Plants that overexpressed either *PpRSL1* or *PpRSL2* were indistinguishable from wild type (Table 1 and see Fig. S1 in the supplementary material). This suggests that neither expression of *PpRSL1* nor *PpRSL2* individually is sufficient for rhizoid development. To determine whether the combined activities of *PpRSL1* and *PpRSL2* were sufficient for moss rhizoid system development, we generated transgenic plants in which both *PpRSL1* and *PpRSL2* were ectopically expressed at high levels in the same plant. These plants accumulated higher steady state levels of both *PpRSL1* and *PpRSL2* transcript than wild type (see Fig. S1C in the supplementary material). Gametophores are multicellular shoot-like axes with an apical cell and lateral leaf-like appendages that develop from buds that form on filamentous protonema in three-week-old wild type (Fig. 1A) (Sakakibara et al., 2003). By contrast, buds were converted into masses of...
rhizoids in plants that overexpress both \(PpRSL1\) and \(PpRSL2\) (Fig. 1E). Because most cells in the buds that overexpressed both \(PpRSL1\) and \(PpRSL2\) differentiated into rhizoids, few or no green gametophores developed in these cultures (Fig. 1D,H); 42.07±5.46 (s.d., \(n = 10\)) gametophores cm\(^{-2}\) developed from wild-type protonema whereas 0.68±0.79 (s.d., \(n = 12\)) gametophores cm\(^{-2}\) developed from protonema that overexpressed both \(PpRSL1\) and \(PpRSL2\) (Table 1). The few gametophores that formed in plants overexpressing both \(PpRSL1\) and \(PpRSL2\) developed many more rhizoids than wild type and these rhizoids developed from cells in the epidermis (Fig. 1B,C,F,G,I-L). Together these data indicate that the combined expression of both \(PpRSL1\) and \(PpRSL2\) is sufficient for the formation of the \(P. patens\) rhizoid system.

**\(PpRSL1\) and \(PpRSL2\) are required for the development of both basal and mid-stem rhizoids**

In wild type, rhizoids differentiate on gametophores. Two populations of morphologically identical rhizoids can be distinguished by the position on the gametophore from which they develop (Sakakibara et al., 2003). The first population of rhizoids form early in gametophore development; they differentiate soon after the gametophore progenitor cell divides to form a bud and continue to develop from epidermal cells below juvenile leaves near the base of the young gametophore (Fig. 2A). When viewed in transverse section, these rhizoids can be seen to grow out from any epidermal cell in the gametophore. Because these rhizoids form at the gametophore base, they are known as basal rhizoids. By contrast, mid-stem rhizoids differentiate from specialized epidermal cells located just below adult leaves on the gametophore axis (leaves 1-5 are considered juvenile and adult leaves are those that form after leaf

**\(PpRSL1\) and \(PpRSL2\) are expressed in cells that form rhizoids**

As \(PpRSL1\) and \(PpRSL2\) are necessary and sufficient for the development of both classes of rhizoids we hypothesized that \(PpRSL1\) and \(PpRSL2\) would be expressed in the cells that give rise to rhizoids. To test this hypothesis, we made \(PpRSL1\) and \(PpRSL2\) double mutants (Fig. 2B,C,F,G), but only few very short basal rhizoids and no mid-stem rhizoids developed in \(PpRSL1\) \(PpRSL2\) double mutants (Fig. 2D,H). The internal cellular anatomy of \(PpRSL1\) \(PpRSL2\) double mutant gametophores was identical to wild type when viewed in transverse section despite the obvious defects in rhizoid development (see Fig. S2 in the supplementary material). This indicates that \(PpRSL1\) and \(PpRSL2\) are required for the development of both basal and mid-stem rhizoids and are not required for the development of other cell types in the gametophore.

**Fig. 4. Auxin positively regulates the transcriptional expression of \(PpRSL1\) and \(PpRSL2\) genes in \(P. patens\).** (A) RT-PCR analysis indicates that a one-week auxin treatment (1 \(\mu\)M NAA) increases steady state levels of \(PpRSL1\) and \(PpRSL2\) in four-week-old gametophores. (B) 1 \(\mu\)M NAA treatment increases the expression levels of \(PpRSL1\) promoter::GUS and \(PpRSL2\) promoter::GUS compared with untreated controls.

**Fig. 5. The spatial pattern of GmGH3 promoter activity in \(P. patens\) gametophores.** (A,B) NAA treatment increases GmGH3 promoter activity in gametophores. GmGH3 expression pattern in untreated controls (A) and after 1 \(\mu\)M NAA-treatment (B) is shown. (C,D) The spatial pattern of GmGH3 promoter activity in basal and mid-stem region of the gametophores. (C) GmGH3 promoter is active in most cells in the epidermis of basal region. (D) GmGH3 promoter activity is higher in the epidermal cells that give rise to rhizoids cells located near leaf trace cells (arrow) in the mid-stem region. Scale bars: 500 \(\mu\)m in A,B; 100 \(\mu\)m in C,D.
the gametophore can develop rhizoids (Fig. 3C,D,G,H). Later in gametophore development, PpRSL1 expression was restricted to the specialized epidermal cells that give rise to mid-stem rhizoids (Fig. 3I-M). By contrast, PpRSL2 was expressed throughout the gametophore epidermis in the regions where mid-stem rhizoids develop (Fig. 3N-R). However, no PpRSL2 expression was detected in other gametophore cell types. Taken together these data indicate that rhizoids develop from cells in which both PpRSL1 and PpRSL2 are expressed, where their joint expression is sufficient to promote the rhizoid development programme.

**Auxin positively regulates cell specific PpRSL1 and PpRSL2 gene expression**

Auxin positively regulates rhizoid development (Ashton et al., 1979; Sakakibara et al., 2003). Because the expression of PpRSL1 and PpRSL2 is sufficient to promote rhizoid differentiation, we hypothesized that auxin controlled rhizoid development by regulating the expression of PpRSL1 and PpRSL2. To test this hypothesis, we determined the effect of auxin-treatment on PpRSL1 and PpRSL2 expression. Auxin-treatment increased PpRSL1 and PpRSL2 steady state mRNA levels (Fig. 4A). To verify independently that auxin positively regulates PpRSL1 and PpRSL2 expression, we determined the effect of auxin-treatment on PpRSL1promoter::GUS and PpRSL2promoter::GUS expression. Auxin-treatment increased the expression of each reporter gene (Fig. 4B).

Furthermore, whereas expression was restricted to those epidermal cells that gave rise to rhizoids in untreated controls, auxin-treatment induced GUS expression along the entire length of the rhizoid in plants transformed with the PpRSL1promoter::GUS and PpRSL2promoter::GUS transgenes. This indicates that not only does auxin-treatment increase PpRSL1 and PpRSL2 transcription but it also extends the expression of these genes into the rhizoids. Together these data demonstrate that auxin positively regulates the expression of PpRSL1 and PpRSL2.

**Rhizoid-forming cells are sensitive to auxin**

Because auxin positively regulates PpRSL1 and PpRSL2 expression, we hypothesized that the cell-specific expression of these genes could be, at least in part, determined by cell-specific sensitivity to auxin. Although we cannot directly assay sensitivity of epidermal cells in the gametophore, we can use a promoter that is transcriptionally responsive to auxin to identify cells that are relatively sensitive to auxin. The expression patterns of the PpRSL1 and PpRSL2 genes were, therefore, compared with the expression pattern of the auxin sensitive GmGH3 promoter, which is restricted to the sites of auxin sensitivity or auxin accumulation in P. patens (Fig. 5A,B) (Rose and Bopp, 1983; Bierfreund et al., 2003; Ludwig-Müller et al., 2009; Fujita et al., 2008; Eklund et al., 2010a; Eklund et al., 2010b). The expression patterns of GmGH3promoter::GUS and PpRSL1promoter::GUS and PpRSL2promoter::GUS were almost identical.

GmGH3promoter::GUS was expressed early in the development of gametophores when the first rhizoids developed and in the cells that gave rise to basal rhizoids in young gametophytes. Later in gametophore development, GmGH3promoter::GUS was active in the epidermal rhizoid progenitor cells that form mid-stem rhizoids like PpRSL1promoter::GUS (Fig. 5C,D). These results indicate that PpRSL1 is most highly expressed in cells that are either relatively sensitive to auxin or accumulate relatively more auxin than surrounding cells. These data are consistent with a model in which the cellular pattern of auxin sensitivity or accumulation regulates the expression of PpRSL1 and PpRSL2, which in turn positively regulates rhizoid development.

**Auxin-induced rhizoid development requires PpRSL1 and PpRSL2 activity**

To verify that auxin controls the development of rhizoids by regulating PpRSL1 and PpRSL2 function, we determined the sensitivity of Pprsl1 single, Pprsl2 single and Pprsl1 Pprsl2 double mutants to auxin-treatment. Auxin-treatment of wild type, Pprsl1 single and Pprsl2 single mutants induced the development of large numbers of rhizoids on gametophores compared with untreated controls (Fig. 6 and see Fig. S4 in the supplementary material). By contrast, auxin treatment of Pprsl1 Pprsl2 double mutants did not increase the number of rhizoids on gametophores compared with untreated controls; the phenotypes of treated and untreated Pprsl1 Pprsl2 double mutant were identical (Fig. 6). The double mutant is, therefore, resistant to the rhizoid-inducing effect of auxin-treatment. This indicates that auxin controls rhizoid development by positively regulating the activity of PpRSL1 and PpRSL2 genes.
Auxin does not regulate AtRHD6 and AtRSL1 during root hair development in A. thaliana

Because auxin controls rhizoid development in moss by positively regulating PpRSL1 and PpRSL2 activity, we determined whether auxin controlled root hair development in A. thaliana by the same mechanism. AtRHD6 and AtRSL1 are the A. thaliana genes that are most closely related to PpRSL1 and PpRSL2 and they positively regulate root hair development; root hairs do not develop in Atrhd6-3 Atrsl1-1 double mutants (Menand et al., 2007) (Fig. 7A). We used RT-PCR to determine whether auxin controlled the expression of AtRHD6 and AtRSL1. Steady state levels of AtRHD6 and AtRSL1 mRNA were indistinguishable in NAA-treated and untreated controls (Fig. 7B). To confirm independently that auxin does not positively regulate AtRHD6 and AtRSL1 activity, we determined whether NAA-treatment altered the abundance of AtRHD6 and AtRSL1 proteins compared with untreated controls. Plants transformed with AtRHD6promoter:GFP:AtRHD6 and AtRSL1promoter:GFP:AtRSL1 protein fusion constructs were treated with NAA. No distinct change in GFP intensity was observed between NAA-treated and untreated controls (Fig. 7C). These data suggest that auxin-regulated development of root hairs is independent of AtRHD6 and AtRSL1.

If AtRHD6 and AtRSL1 act independently to control root hair development, we predicted that NAA-treatment of Atrhd6-3 Atrsl1-1 would suppress the hairless phenotype characteristic of this double mutant. As predicted, NAA-treatment induced root hair development in the double mutant (Fig. 7D) (Yi et al., 2010). Furthermore, auxin-treatment of the Atrhd6-3 Atrsl1-1 double mutant induced the expression of the root hair specific AtEXP7promoter:GFP gene, which is not expressed in untreated Atrhd6-3 Atrsl1-1 controls (Fig. 7E). This indicates that the induction of AtEXP7 expression by auxin is independent of AtRHD6 and AtRSL1 activity. Taken together these data indicate that auxin does not act through AtRHD6 and AtRSL1 during root hair development in A. thaliana, in contrast to P. patens where auxin controls rhizoid development by positively regulating PpRSL1 and PpRSL2 expression. This suggests that the mechanism by which auxin regulates rhizoid and root hair development is different in P. patens and A. thaliana and implies that this mechanism has changed since these two species last shared a common ancestor over 400 million years ago.

DISCUSSION

We showed here that PpRSL1 and PpRSL2 genes are necessary and sufficient for the development of multicellular rhizoids in P. patens. This suggests that PpRSL1 and PpRSL2 are key regulators of rhizoid development in early diverging groups of land plants. As RSL genes also control the development of single celled root hairs in angiosperms, we propose that RSL genes were co-opted to control the development of filamentous cells (root hairs) in roots when these specialized axes evolved in later diverging groups of land plants (lycophytes, monilophytes and seed plants). Therefore, it is likely that RSL genes regulate the development of filamentous cells at the plant-soil interface in all groups of land plants. This hypothesis is supported by the observation that RSL genes have been found in all land plants for which genome sequence is available (Pires and Dolan, 2010).

Although auxin positively regulates root hair and rhizoid development in A. thaliana and P. patens, respectively, our data suggest that the mechanism of auxin action differs between these species in at least two ways (Ashton et al., 1979; Pitts et al., 1998; Sakakibara et al., 2003; Cho et al., 2007; Jones et al., 2009). Comparison of amino acid sequences shows that PpRSL1 and

Fig. 7. Auxin-control of root hair development is independent of AtRHD6 and AtRSL1. (A) Tree showing the relationships between A. thaliana and P. patens RSL proteins. AtRHD6 and AtRSL1 are the A. thaliana RSL proteins that are most closely related to PpRSL1 and PpRSL2. (B) RT-PCR showing that steady state levels of AtRHD6 and AtRSL1 mRNA remain unaltered by auxin treatment (150 nM NAA). (C) Levels of GFP:AtRHD6 and GFP:AtRSL1 fusion proteins are not regulated by auxin. AtRSL1promoter:GFP:AtRSL1 and AtRHD6promoter:GFP:AtRHD6 are shown with (right) or without (left) treatment with 150 nM NAA. (D) Auxin treatment induces root hair development on Atrhd6-3 Atrsl1-1 double mutants whereas untreated controls remain hairless. (E) AtEXP7promoter:GFP expression is induced by auxin. There is no AtEXP7promoter:GFP expression in untreated controls.
PpRSL2 are most closely related to AtRHD6 and AtRSL1 in A. thaliana, which control the development of root hairs; AtRHD6 AtRSL1 double mutants do not develop root hairs (Menand et al., 2007). Auxin promotes rhizoid development by positively regulating PpRSL1 and PpRSL2 genes in P. patens. By contrast, we can find no evidence for the regulation of AtRHD6 and AtRSL1 by auxin. Instead, auxin promotes root hair development by positively regulating the expression of a gene from a different clade called AtRSL4, which acts downstream of AtRHD6 and AtRSL1 (Yi et al., 2010). This indicates that although the mechanisms that control the development of filamentous cells at the land plant-substrate interface is ancient, changes in the regulatory interactions between components of this network have occurred during the course of land plant evolution.

A second difference in auxin-regulated development of root hairs and rhizoids is highlighted by the demonstration that the auxin sensitive reporter gene GmGH3 promoter:GUS is expressed in cells that go on to form rhizoids. This suggests that relatively high levels of auxin might accumulate in these cells compared with the surrounding epidermal cells or that the rhizoid-forming cells are more sensitive to auxin than the surrounding cells that do not form rhizoids. We propose that this relatively high level of auxin or high level of auxin sensitivity then activates the expression of PpRSL1, which then positively regulates rhizoid differentiation. This contrasts with A. thaliana, in which the auxin sensitive reporter DR5-GFP is relatively more active in the non-hair cells and less active in the cells that form root hairs, which has been interpreted to mean that the non-hair cells (N cells) of the A. thaliana root are more sensitive to auxin than the hair cells (H cells) (Jones et al., 2009).

Together these data indicate that although auxin regulates RSL networks in P. patens and A. thaliana, its interaction with these networks is different in each species. If the moss RSL network represents the ancestral state that existed in early diverging groups of land plants, our data suggest that the way in which auxin controls the network changed during land plant evolution. Nevertheless, our results indicate that RSL genes have played a pivotal role in controlling the development of the filamentous cells at the plant-soil interface in gametophytes and sporophytes since plants appeared on land in the Ordovician Period over 460 million years ago (Kenrick and Crane, 1997; Wellman and Gray, 2000; Wellman et al., 2003).

Because auxin-regulated RSL networks control the development of tip growing rhizoid and root hair cells in mosses and flowering plants, which last shared a common ancestor over 420 million years ago, it can be concluded that the RSL-mechanism is ancient. It is, therefore, likely that auxin-regulated RSL genes control the development of rhizoids and root hairs in lycopodites, monilophytes and seed plants. Furthermore, because monilophytes (ferns and horsetails) develop both root hairs and rhizoids in the sporophyte and gametophyte generations, respectively, these data also predict that auxin-regulated RSL genes control the development of these cell types in the two stages of the life cycle of these plants (Bower, 1929). Consistent with these predictions is the demonstration that auxin positively regulates the development of rhizoids and root hairs in the gametophytes and sporophytes, respectively, in ferns (Alsopp, 1952; Bloom and Nichols, 1972; Hickock and Kiruluk, 1984). Nevertheless, RSL genes have yet to be identified in monilophytes. However, this is likely to be due to a lack of genome and expressed sequence tags sequences among this group.

Auxin positively regulates rhizoid development in Marchantia polymorpha, an extant representative of the earliest diverging group of land plants, the liverworts (Kaul et al., 1962). Furthermore, land plants and charophycean algae are derived from a common ancestor that existed some time before 450 million years ago. Given that some charophycean algae such as Nitella species and Chara species develop rhizoids it is possible that the mechanism controlling land plant and algal rhizoid development might be derived from the same ancestral mechanism. Although no genes controlling the development of charophycean rhizoids have been identified, auxin positively regulates rhizoid development in Chara species and other green algae (Klämbt et al., 1992; Jacobs, 1951). It is, therefore, possible that RSL-like genes are positively regulated by auxin during the development of algal rhizoids. If this were the case it would suggest that the mechanism controlling the development of filamentous anchoring cells is conserved among land plants and related algae. Alternatively, although auxin positively regulates the development of filamentous cells in land plants and algae, it is possible that auxin activates different downstream regulatory networks that control the development of filamentous anchoring cells in algae and land plants. Distinguishing between these alternatives requires the characterization of the genetic network that controls the development of algal rhizoids.

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Competing interests statement

The authors declare no financial interests.

Supplementary material

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References


Early land plant rhizoid development


