

Poster Abstracts

P1: Diurnal expression of the *Lhcb* gene in the moss *Physcomitrella patens*.

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Many researchers reported that *Lhcb* genes, encoding chlorophyll-binding proteins, from various higher plants exhibited robust oscillations with daily intervals. These rhythms are controlled by the circadian clock, a self-sustained oscillator with a period of about one day, and persist even in constant conditions such as continuous light (LL).

We investigated whether or not the *Lhcb* genes of the moss *P. patens* are also under the control of the circadian clock. First, we isolated two *Lhcb* genes, tentatively named as *PpLhcb1* and *PpLhcb4*, by a screen of a *P. patens* genomic DNA library. The two genes clustered in a branch very close to, but distinct from, the *Lhcb1* and *Lhcb2* groups in higher plants in the phylogenetic tree constructed with *Lhcb* sequences from several plant species. Northern blotting analyses using the *Lhcb4* coding region as a probe revealed the expression pattern of *Lhcb* genes under light-dark cycle (LD), LL and continuous dark (DD) conditions. We observed very robust oscillations and damping oscillations with daily intervals under LD and DD conditions, respectively, whereas we could not observe rhythmic expression under LL condition. Some characteristics of the rhythms in LD and DD conditions suggested that the moss *Lhcb* genes are also under the control of the circadian clock. We fused the putative promoter region of *Lhcb4* upstream of the firefly luciferase gene, and introduced this fusion DNA into the moss genome by the PEG-mediated transformation. The resulting transformants exhibited bioluminescence whose temporal profiles were consistent with the Northern data. We are now investigating light resetting properties of the damping oscillation of the *Lhcb4* gene in DD.

P2: Evolution of plant circadian clocks.

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Circadian clocks are 24-hr biological oscillators that allow organisms to anticipate predictable diurnal changes in environmental conditions. Recent work in my laboratory and others identified some of the key components of the circadian system of *Arabidopsis*. As in *Drosophila*, mammals, fungi and cyanobacteria, the core mechanism of the plant circadian clock is thought to consist of a transcriptional feedback loop. During the day, the MYB transcription factors LHY and CCA1 repress expression of the *TOC1* gene, encoding an atypical response regulator protein. Reduced expression of LHY and CCA1 at night allows expression of *TOC1*, which in turn promotes transcription from the LHY and CCA1 promoters at dawn. This regulatory feedback loop is entrained to environmental cycles through the action of multiple photoreceptors (phytochromes and cryptochromes). The clock feeds back on these light input pathways to modulate responsiveness with the time of the day. Thus expression of multiple elements of phototransduction pathways (including the photoreceptors themselves) exhibits circadian rhythmicity, and the contribution of these additional oscillatory feedback loops to the overall rhythmicity is not clear.

Comparison of circadian clock mechanisms between *Drosophila* and mammals, as well as between species of insects has revealed conserved aspects as well as striking differences. In order to identify an ancestral and possibly simpler form of the plant circadian clock, we now wish to investigate the molecular mechanism of the circadian clock in a primitive land plant. The moss, *Physcomitrella patens*, is an attractive system because of its easy transformation and of gene targeting possibilities. So far we have demonstrated that expression of the *Chlorophyll a/b-binding protein (CAB)* gene exhibits circadian rhythmicity in moss. We have identified sequences in EST databases that show homologies to components of the *Arabidopsis* clock. We plan to systematically knock-out and overexpress these proteins in order to test whether their loss-of function or misexpression results in aberrant rhythmic expression of CAB.

P3: Functional Analysis of the *Physcomitrella patens mago nashi*

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Maternal effect alleles of the *Drosophila melanogaster mago nashi* gene have embryo patterning phenotypes caused by the mis-localisation of RNA species within the oocyte. These alleles also cause a disorganisation of the microtubule cytoskeleton, suggesting that *mago nashi* could be involved in a fundamental mechanism of *Drosophila* cell polarity establishment. The study of plant homologues of *mago nashi* might reveal some mechanisms of plant cell polarity regulation. An *Arabidopsis mago nashi* EST clone was used to isolate a *Physcomitrella patens mago nashi* cDNA from an aba-induced cDNA library. The probing of *Physcomitrella* genomic Southern blots suggested that the cDNA represents a single copy locus. The genomic *mago nashi* was cloned, and an *nptII* kanamycin-resistance gene was inserted into the genomic fragment, creating a plasmid to knockout the *mago nashi* locus. Protoplasts were transformed using linearised plasmid. A total of 87 stable transformants were recovered. 6 of 20 stable mutants analysed using PCR were identified as having integrations at the *mago nashi* locus. Preliminary phenotypic analysis has shown that some transformants exhibit a protonemal phenotype. Gamete development may also be affected.

P4: Overexpression screening of genes involved in asymmetric cell division in the moss *Physcomitrella patens*

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Asymmetric cell division is of fundamental importance in the generation of the overall cellular pattern. Its molecular mechanism, however, remains unknown in plants. The moss *Physcomitrella patens* will be useful to dissect this molecular mechanism, not only because its body plan is relatively simple, making a study at a single cell level feasible, but also because it is the only plant in which gene targeting exhibits a high rate of success. Regeneration of moss protoplasts assures a good system for the study of several interesting facets such as cell polarity, asymmetric cell division, and cell differentiation. We have devised a comprehensive system to screen genes affecting the regeneration step of protoplasts in *P. patens*.

We constructed three kinds of full-length cDNA libraries based on biotinylated cap trapper method from non-treated, auxin-treated, and cytokinin-treated protonemal cells of *P. patens*, then determined the sequences from 5' and 3' ends of cDNA clones from each library. The sequence data were clustered, annotated by BLAST search and more than 50,000 ESTs were deposited in public database. Sequence analyses of the ESTs revealed that about 93% of our cDNA clones appeared to cover the complete coding region, suggesting that these will serve as a good source of full-length clones for functional analysis of genes and their products. We used these clones as materials for overexpression screening. Individual cDNAs were selected based on their sequence, subcloned under a constitutive promoter and introduced into moss protoplasts for transient expression. We scored the phenotypes of regeneration of the protoplasts and found that some clones caused the delay of regeneration. About 1% of cDNAs exhibited aberrant cell shapes when overexpressed, some of which encode cytoskeletal proteins and unknown proteins. We will present the current status of this project.

P5: Ethylene functions in the moss, *Physcomitrella patens*.

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Ethylene is one of the typical plant hormones that work in many processes, such as seed germination, root hair development, fruit ripening and stress responses in higher plants. The putative ethylene receptor gene, ETR1 was first isolated in *Arabidopsis* and its homolog is found in many higher plants now. The sequence of ETR1 resembled the prokaryotic two-component sensory His kinase and the N-terminus of Etr1 protein was found to bind directly to ethylene molecule. To date, there is no clear evidence that lower plants utilize ethylene in some way, and ethylene is thought to be the plant hormone only working in higher plants.

We have isolated the putative ETR1 gene from the moss, *Physcomitrella patens* genome and named the gene as PpETR1. To investigate its function, we first over-expressed PpETR1 and its truncated form in *P.patens* protoplasts. While neither the full size PpETR1 gene, expressed under the control of the CaMV35S promoter, nor the C-terminally truncated form inhibited protoplast growth, the N-terminally truncated form of PpETR1 had a severe inhibitory effect.

Because a dominant negative *etr1-1* mutant and its putative downstream *ctr1* mutant of *Arabidopsis* were sensitive to high osmolality, we considered the reason of inhibitory effect of the N-terminal segment of PpETR1 might be through osmotic status of the protoplast embedded in 8% mannitol. So we next investigated ethylene function in *P. patens* using the well-known precursor 1-aminocyclopropane-1-carboxylic acid (ACC), its competitive inhibitor aminooxyacetic acid (AOA), and silver nitrate which is thought to inhibit ethylene perception process of Etr1 protein through its copper binding domain.

When cultured on a plate, 50 μ M of ACC seemed to have no clear effect as was reported previously. When cultured in liquid medium in a closed bottle, ACC did not inhibit growth at all and many gametophytes were produced, but growth in liquid medium in air was inhibited by ACC. When tissues were transported from plate to liquid medium with 15 μ M AOA or 1 μ M silver nitrate, they all died soon.

These results indicated that *P. patens* utilized ethylene in order to adapt to the external osmotic status, and this function is especially important for growing from water into the air. Considering the reports that ACC synthetase changed its activity in relation to the external osmotic status in tomato and tobacco cultured cells, this ethylene function of regulating osmotic status may be common in higher and lower plants. And, since there was no report of ethylene function in algae, ethylene may have played an important role for the colonization of land by plants.

P6: Cell wall biosynthesis and control of morphogenesis in *Physcomitrella patens*

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Two types of growth coexist in moss: tip growing protonema filaments and more complex tri-dimensional gametophores. The growth transition is initiated by the formation of buds. In higher plants it has been suggested that cell wall organisation could play a role in the control of plant morphogenesis and architecture.

In bryophytes the composition as well as the organisation of cell wall polysaccharides is poorly known. Cellulose synthase rosettes have been evidenced on the plasma membrane of caulonema cells in *Funaria hygrometrica*. Moreover moss EST databases contain sequences homologous to higher plant genes involved in the cellulose synthesis and cell wall organisation.

We have obtained preliminary data on the *P. patens* cell wall by FTIR-microspectroscopy analysis and by determination of the sugar composition by Gas Liquid Chromatography. The composition of the total cell walls is close to that observed for cell walls from dicotyledonous plants. Isolation and analysis of individual wall polysaccharides is under way.

Moreover two cDNA encoding protein reminiscent of plant structural cell wall proteins have been isolated among genes specifically expressed during bud formation in response to cytokinin. The expression pattern of these genes will be discussed and knockout experiments described.

P7: Structural characterization of MADS-box genes in the moss *Physcomitrella patens*

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MADS-box genes play important roles in the formation of flowers within angiosperms, as well as in the formation of reproductive structures within gymnosperms. In contrast, almost nothing is known about the function of MADS-box genes within more basal tracheophytes such as ferns, or within non-vascular plants. Therefore, we decided to study the function of MADS-box genes within the moss *Physcomitrella patens*, which is the only land plant known so far that offers the possibility to efficiently knock out genes via homologous recombination.

The MADS-box genes of *P. patens* represent MIKC-type genes previously known only from seed plants and ferns. By comparison of sequence similarities and by studying exon/intron structures the *P. patens* MADS-box genes can be divided into two types, termed MIKC^c- and MIKC^{*}-type. The MIKC^c-type genes of *P. patens* *PPM1*, *PPM2* and *PpMADS1* have nearly identical structures as the MIKC^c-type genes from higher plants. They share a very similar exon/intron structure, the conserved pattern of hydrophobic aminoacids within K-domains, as well as the diagnostic length of the fifth and sixth exon of 42 bp. The I-domain is placed on one exon.

In contrast, the MIKC^{*}-type genes *PPM3*, *PPM4*, *PpMADS2* and *PpMADS3* differ from this conserved structure by longer I-domains distributed on four or five exons, respectively. It was shown that the MIKC^{*}-type genes are not restricted to the moss *Physcomitrella patens*. Also *LAMB1* from the lycopod *Lycopodium annotinum* shows this longer I-region consisting of four exons instead of one. That *LAMB1* belongs to the MIKC^{*}-type genes is also supported by phylogeny reconstructions, with the MIKC^{*}-type genes forming a clade supported by high bootstrap values. Due to the isolation of MIKC^c- and MIKC^{*}-type genes from *P. patens* and from *L. annotinum*, the last common ancestor of mosses and lycopods about 450 MYA must have had at least one MIKC^c- and one MIKC^{*}-type gene.

Evidence was gained that gene networks similar to those known from higher plants may also exist in mosses. This is supported by the appearance of regulatory promoter elements, the high number of at least five MADS-box genes of MIKC^c- and MIKC^{*}-type each, and the existence of K-domains in the predicted gene-products, which are necessary for protein dimerization.

P8: Expression analysis of PpSig1, PpSig2 and PpSig5 genes encoding putative plastid RNA polymerase sigma factors in the moss *Physcomitrella patens*.

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Expression of plastid genes is controlled at the transcriptional level in response to developmental and environmental signals. Transcription in plastids is accomplished by two distinct RNA polymerase enzymes, one of which resembles eubacterial RNA polymerases in both subunit structure and promoter recognition properties. The holoenzyme of this eubacteria-type RNA polymerase contains a catalytic core composed of plastid-encoded subunits, assembled with a nuclear-encoded promoter-specificity factor, sigma. Recently , families of sigma-like factor genes were identified from several higher plants. In our laboratory ^{1,2}, we identified *PpSig1* and *PpSig2*, sigma-like factor genes in *Physcomitrella patens*, and demonstrated that the transcription of both genes was induced by light. We have recently succeeded in cloning a new sigma-like factor gene in *Physcomitrella patens*. By a phylogenetic analysis and a comparison of intron positions , this gene was classified into Sig5 group of higher plant sigma-like factors. Thus, we named this gene *PpSig5*. We are currently investigating the expression patterns of *PpSig1*, *PpSig2* and *PpSig5* genes under a light/dark cycle (12 hr light,12 hr dark)

1. Hara, K., Sugita, M., Aoki, S. (2001) *Biochim. Biophys. Acta* 1517 (82), 302-306
2. Hara, K., Morita, M., Takahashi, R., Sugita, M., Kato, S., Aoki, S. (2001) *FEBS Letters* 499 (81-2) 87-91

P9: Identification of gametangium-specific gene trap lines in the moss *Physcomitrella patens*.

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In land plants, both sporophyte and gametophyte form reproductive organs. In the course of the land plant evolution, the size and the complexity of sporophytic reproductive organ were increased, and those of gametophytic organ were decreased. The relationship between the evolution of sporophytic reproductive organs and the evolution of genetic network including MADS-box genes has been discussed. On the other hand, the evolution of developmental mechanisms for the gametophytic reproductive organs is unclear, because the mechanisms have been studied only on the angiosperms. It is necessary to know the developmental mechanism of the gametophytic organs in bryophytes or pteridophytes that form antheridia and archegonia as the gametophyte reproductive organs. *Physcomitrella patens* is a model bryophyte for which a number of gene trap lines have already been established, and therefore useful to isolate genes specifically expressed in gametangia.

To study the developmental molecular mechanisms of antheridia and archegonia, and then to compare them to the developmental mechanisms of pollen and embryo sac of the angiosperms, we explore genes involved in archegonia and/or antheridia formation, using the gene trap lines of *P. patens*. We screened approximately 3300 gene trap lines and obtained more than 250 lines with archegonia and/or antheridia specific gene expression patterns. Some lines showed cell or tissue specific expression patterns, such as in an egg cell or an archegonial neck cell. There were also the lines with expression signals in the primordial cells of gametangia, showing the presence of possible trapped genes involved in gametangia determination.

P10: Sequence Clustering of *Physcomitrella* ESTs and Comparison to Higher Plants

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The *Physcomitrella* EST Project (PEP) has been in place in Leeds since 1999. PEP has aimed to describe the transcriptome of moss *via* sequencing of ESTs obtained from tissue grown with different hormone treatments.

Bioinformatics techniques have been developed and applied to effect a computerised normalisation of *Physcomitrella* ESTs *via* clustering and consensus generation. Of the 14044 currently-sequenced ESTs, we have generated 8834 individual consensi.

Sequence comparison to the *Arabidopsis* and *Oryza* ORFeomes has been carried out using BLASTX has shown that 65% of these contigs have homologs in *Arabidopsis* at E values equal to or less than 0.001 and that 44% have homologues in *Oryza* at the same level. Analysis of the length of these matches has shown that over 70% of these have a homology length of over 200bp.

TBLASTN searches against the *Physcomitrella* consensi database have shown that 44% of the *Arabidopsis* ORFeome and 31% of the *Oryza* ORFeome are now represented in the PEP consensus sequence database.

Strategies are being developed and applied to select clones of interest for microarray expression studies. Results from these analyses are being made available at the PEP website: <http://www.moss.leeds.ac.uk> .

P11: Isolation of NAC genes in green algae and *Physcomitrella*: origin and diversification of NAC gene family.

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The shoot apical meristem in the sporophyte (diploid) generation is an outstanding feature of vascular plants, which enables indeterminate growth to have a large body and to produce many spores or seeds. In contrast, bryophytes form only a single sporangium and do not have a shoot apical meristem that proliferate organs in the sporophyte generation. Is this because bryophytes do not have homologues to the genes required for the shoot apical meristem formation in angiosperms? Or do have these genes, but the genes are regulated to form a single sporangium? *CUC1* and *CUC2*, which belong to the NAC gene family, are candidate genes that may have played a key role in the evolution of meristem, as they are essential for the formation of shoot apical meristem and define organ boundaries in *Arabidopsis* and their homologues are reported only in Angiosperms. We cloned 23 NAC genes in *Physcomitrella*, one gene each in *Coleochaete* and *Closterium*. Based on a phylogenetic analysis, NAC gene family originated before the split of *Closterium* (Zygnematales) and the land plants lineage, and diversified to at least four members in the last common ancestor of mosses and vascular plants. One of these descended to *CUC1* and *CUC2* in *Arabidopsis* and to a group of genes in *Physcomitrella*, which are highly similar to *CUC1* and *CUC2*. We expect that the *Physcomitrella* genes function in the sporophyte development and can play the role that *CUC1* and *CUC2* have in the shoot apical meristem formation.

P12: Isolation and knockout of the MSH2 gene in *Physcomitrella patens*

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In the moss *Physcomitrella patens*, integrative transformants from homologous recombination are obtained at an efficiency comparable to that found for yeast. This property, unique in the plant kingdom, allows the knockout of specific genes. It also makes the moss a relevant model to study homologous recombination in plants. We previously isolated the *P. patens* MSH2 (PpMSH2) cDNA (Brun *et al.* , 2001). In eukaryotes the MSH2 protein play a critical role in homologous recombination and in the detection of mismatch between sequences. Here we describe the characterisation of the PpMSH2 gene and its knockout. The molecular characterisation of the knockout transformants will be presented. The ability of the *Ppms2* mutants to perform homologous recombination will be tested. For this purpose we are using a construct containing a partial APT (adenine phosphoribosyl transferase) gene disrupted by a dominant marker (neomycin) as a reporter of the targeting efficiency. *Apt* disruptants can be identified by growth on 2-fluoroadenine (2-FA), a toxic compound for wild-type mosses. Targeted integration frequency can be measure by number of 2-FA resistant colony versus neomycin resistant colony. In second time, and in order to test the role of PpMSH2 in homologous recombination, we will use a mutated version of the APT construct showing mismatch with the resident APT gene.

P13: Cloning and analysis of glycine-rich RNA binding protein in *Physcomitrella patens*

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Glycine-rich RNA-binding proteins (GRP) have been identified in a number of plants and animals. Most of them have on their N-terminus RNA recognition motif (RRM), which is RNA binding domain, and on their C-terminus glycine-rich domain. It has been suggested that some may be involved in stress response, as their mRNA accumulation level was modified following exposure to cold, wounding, acute hypersensitive response, ABA treatment, salicylic acid treatment, or water stress. For example, GRP homologues in *Arabidopsis* (*AtGRP7* and *AtGRP8*) are regulated by low temperature as well as circadian clock. But the physiological function of GRP remains unknown. To analyze the role of GRP by gene disruption, we characterized the cDNAs of GRP in *Physcomitrella patens*.

Three full-length cDNA clones each encoding a putative GRP were isolated from a cDNA library prepared from polyA⁺ RNA from 7 day old protonemata of *P. patens*. They were named *PpGRP1*, *PpGRP2* and *PpGRP3*, which encode putative polypeptides of 162, 178 and 155 residues, respectively. The RRM regions of *PpGRP1* and *PpGRP2* were, respectively, 74% and 71% similar to the RRM of *AtGRP7*. Preliminary genomic sequencing suggested that the positions of three introns in *PpGRP3* is similar to those of introns in *Arabidopsis* GRP genes. *PpGRP3* had a putative transit sequence. The protein-sGFP fusions of *PpGRP1* and *PpGRP2* were targeted to the cell nucleus, while that of *PpGRP3* was targeted to mitochondria. The level of *PpGRP* transcripts after cold treatments was increased. Treatment with ABA had no significant effect on the level of *PpGRP* transcripts.

P14: Targeted disruption of a hexokinase gene in the moss *Physcomitrella patens* and characterization of the resulting phenotype.

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Hexokinase catalyzes the first step in hexose metabolism, but has also been implicated as being involved in sugar sensing and signalling, which in turn controls a wide variety of developmental and metabolic processes in plants. The molecular details of this postulated regulatory function of plant hexokinases are largely unknown. To learn more about the function of hexokinases in plants we have cloned a hexokinase gene, *PpHKK1*, from the moss *Physcomitrella patens*. In addition to *PpHKK1*, there seem to exist at least two more hexokinase genes in the moss. We found that *PpHKK1* is constitutively expressed in chloronemal tissue. We have used a targeted disruption to knock out *PpHKK1* and are now analyzing the resulting phenotype. We have found that the *hkk1* mutant exhibits increased sensitivity to the plant hormones abscissic acid and cytokinin. There are also indications that the mutant is deficient in the growth response to glucose, which could suggest a possible function for *PpHKK1* in glucose sensing or signalling.

P15: Targeting of a *Physcomitrella patens* Rho-GTPase (rac) gene

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Replacement targeting vectors of a low copy number *Physcomitrella patens* sequence showing homology to the *Arabidopsis thaliana* *Rop* gene sub-family have been constructed. Linear DNA fragments were transformed into *P. patens* to elucidate the target gene function. *Rop* activity, a plant specific Rho-GTPase, has been implicated in tip growth, cell polarity, the actin cytoskeleton, pathogen defence, secondary cell wall formation and meristem signalling. The signalling pathways have not yet been elucidated. Studies of the *Rop1Ps* and *Rop1At* indicated that *Rop*-GTPases act as a central switch for the polar out growth of pollen tubes by coupling spatial control with temporal control (Zeng & Yang, 2000; Li, *et. al.*, 1998). Tip-localized calcium signalling acts downstream of *Rop* to activate tip growth and is controlled by the negative feedback loop components of which include *Rop*-GTPase-activating proteins (*RopGAPs*) and the putative *Rop*-GTPase effector phosphatidylinositol monophosphate kinase (PtdInsPkinase). The formation of pollen tip-focused calcium gradient and the tip-localized calcium influx may therefore be regulated through the *Rop*-Ins(1,4,5) P_3 -Ca²⁺ system by hydrolysis of the PtdInsPkinase and the localized PtdIns(4,5) P_2 to Ins(1,4,5) P_3 (Zheng & Yang, 2000; Li, *et. al.*, 1999; Aspenstrom, 1999).

PCR analysis of a group of randomly selected transgenics revealed that three types of targeting incidence occurred at the targeting locus. The phototropic response to white light of all the transgenics is similar to the wild-type strain at all different light intensities examined. However, only the protonemal filaments of the targeted lines have abnormal shaped chloroplasts when cultures were treated in 2.1 mW/m² white light. Analysis of the lipid profile in the *P. patens* transgenics is discussed.

Zheng, Z-L., and Yang, Z. (2000). The Rop GTPases switch turns on polar growth in pollen. *Trends in Plant Science*. 5: 298-303.

Aspenstrom, P. (1999). Effectors for the Rho GTPases. *Current Opinion in Cell Biology*. 11: 95-102. Li, H., Lin, Y., Heath, R. M., Zhu, M. X., Yang, Z. (1999). Control of pollen tube tip growth by a *Rop* GTPase-dependent pathway that leads to tip localised calcium influx *The Plant Cell*. 11: 1731-1742.

Li, H., Wu, G., Ware, D., Davis, K.R., Yang, Z. (1998). *Arabidopsis* Rho-Related GTPases: Differential Gene Expression in Pollen and Polar Localisation in Fission Yeast *Plant Physiology*. 118: 407-417.

Li, H., Lin, Y., Heath, R. M., Zhu, M. X., Yang, Z. (1999). Control of pollen tube tip growth by a *Rop* GTPase-dependent pathway that leads to tip localised calcium influx *The Plant Cell*. 11: 1731-1742.

P16: The homeodomain-leucine zipper I gene is involved in epidermal cell differentiation in the moss *Physcomitrella patens*

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The differentiation of epidermal cells is important for immobile plants because they are in direct contact with the biotic and abiotic environments. Rhizoids are multicellular filaments that differentiate from the epidermis, and they have similar functions to root hairs in vascular plants in that they support the plant body and are involved in the absorption of water and nutrients. Rhizoids are widely observed in green plants, including pteridophytes, bryophytes, and green algae, but their development has not been studied at the molecular level, mainly because of a lack of useful model systems. The moss *Physcomitrella patens* is a suitable plant in which to study rhizoid differentiation, since techniques for transformation and gene targeting by homologous recombination have been established in this plant during the last decade. The mechanisms underlying rhizoid differentiation in *P. patens* were examined. Two types of rhizoids with distinct differentiation patterns (basal and mid-stem rhizoids) were recognized. The differentiation of basal rhizoids from epidermal cells was induced by exogenous auxin, while that of mid-stem rhizoids required an unknown factor in addition to exogenous auxin. Once an epidermal cell is destined to become a rhizoid initial cell, the expression of the homeodomain-leucine zipper I gene *Pphb7* is initiated. The analysis of *Pphb7* disruptant lines showed that *Pphb7* function was crucial for certain activities and features of the rhizoid cell, which included the induction of pigmentation and the inhibition of chloroplast division and expansion. This is the first report on the involvement of a homeodomain-leucine zipper I gene in epidermal cell differentiation. A model for rhizoid differentiation is proposed.

Sakakibara, K., Nishiyama, T., Kato, M., Hasebe, M. 2001. Isolation of Homeodomain - Leucine Zipper Genes from the Moss *Physcomitrella patens* and the Evolution of Homeodomain-Leucine Zipper Genes in Land Plants. *Mol. Biol. Evol.* 18(4): 491-502.

P17: Identification of putative arabinogalactan protein genes from *Physcomitrella patens*

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Arabinogalactan protein (AGP) is a widely distributed proteoglycan in the plant kingdom. Although AGP is suggested to be involved in differentiation, development and cell-cell communication in plants, its function remains unclear. A reverse genetics approach will give us clues to understand the molecular function of AGP. *Physcomitrella patens* is ideal for this purpose because gene targeting is available with high efficiency in this plant.

In *P. patens* little is known about AGP, and no sequence information has been reported. We performed a BLAST search of the moss EST database by BLAST program using *Arabidopsis* AGP (AtAGP1-17 and AtFLA1-14) genes as queries. So far, we have retrieved 6 cDNAs encoding putative AGP proteins. From the analyses of deduced amino acid sequences, all proteins encoded by these cDNAs appear to be classical AGPs. Classical AGPs are defined by; 1) an N-terminal secretion sequence that is removed from the mature protein, 2) the mature protein contains Pro/Hyp (hydroxyproline), Ala, Ser, Thr and Gly as the major amino acids and 3) the presence of GPI-anchor signal in the C-terminal portion of the ORF.

On a structural basis, *P. patens* AGPs are classified into three subgroups, which are also found in *Arabidopsis*. There is one AGP with a mature protein of 117 amino acids, and three AGP cDNAs encode peptides with short mature proteins of 11, 16 and 17 amino acids, respectively. Another group consists of two AGPs that contain one or two fasciclin domains. Fasciclin domains were first found in the *Drosophila* cell adhesion molecule (CAM) fasciclin I, and it was also reported that algal CAM which contains this domain plays an important role in adhesion of neighboring cells during embryogenesis in *Volvox*.

Southern blot analysis of *P. patens* genomic DNA showed each cDNA exist as a single copy gene. We have started to make transgenic moss lines for over-expression and knockout of these AGPs. Some preliminary results will be presented.

P18: Bryophyte cryopreservation. The influence of pre-culture methods on post-thaw recovery

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The influence of pre-culture media and pre-culture conditions for the cryopreservation of six bryophytes, *Physcomitrella patens*, *Marchantia polymorpha*, *Sphagnum subsecundum*, *Dicranella staphylina*, *Atrichum tenellum*, and *Leptobryum pyriforme* was studied. Chopped plant material was pre-cultured for three days on medium, either free of any supplements or supplemented with abscisic acid, proline, glucose, mannitol or on medium supplemented with dimethylsulfoxide and mannitol. Plants were pre-cultured at 15⁰C and 25⁰C. As culture vessels, petri dishes were compared to cryovials. Regrowth of the plants after freezing and thawing was determined daily by visual evaluation and by fresh mass determination after a post-culture period of 30 days. Dependent on the pre-culture conditions a survival of all species after freezing/thawing was achieved. The different conditions lead to different regeneration efficiency of the species. Also the time until the tissue started to regrow was different. Based on these results specific pre-culture conditions for optimum regenerations of all tested bryophyte species were selected. For that the results of the present study can be used as prerequisite for long term storage of bryophytes, especially for cryopreservation of bryophyte germplasm collections.

This work has been performed in a joint project with BASF Plant Science GmbH.

P19: Flowcytometric analysis of ploidy in *Physcomitrella patens*

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The current production of a saturated mutant collection is based on PEG-mediated protoplast transformation and after regeneration and selection on antibiotic-containing medium the ploidy level of each stable transformant is determined by flowcytometry. The ploidy level of 45,000 transgenic *Physcomitrella* plants has been tested so far and 13.2 +/- 7.8 % were polyploid (2n = 13.0 % and 4n = 0.2 %), probably due to protoplast fusion during the transformation procedure. The percentage of polyploid plants differs a lot between single transformations; however no correlation between preculture or the transformation procedure and the number of polyploid mutants regenerated afterwards could be detected so far. In two experiments we compared two ways of mixing protoplasts and DNA during the transformation by either carefully rotating the glass tubes in our hands or using a vortexer. However the number of polyploid transformants coming out of 2. selection were in the same range.

After quality control (identification of stable transformants by PCR or third selection and determination of ploidy level) moss plants are characterized phenotypically. To check whether variation in moss phenotype could be induced not only by gene knockout but also by changes of the ploidy level, ploidy and phenotype of 418 haploid and 80 diploid untransformed regenerated moss plants were correlated. While most of the haploid regenerants looked like wildtype (> 90 %), around 75 % of the diploid plants showed a phenotype deviating from wildtype. More than 90 % of the haploid plants showed normal growth on Knop medium, compared to only 20 % of the polyploid plants. Changes in the ploidy level did rarely affect the cell form of the gametophores and had no effect on the color of the plant, but many diploid plants showed a reduced number of gametophores (> 90 %) and changes in the form of the gametophores, like double tips (> 20 %) or cell outgrowth (> 5 %). Phenotypic deviations in more than one characteristic strongly indicated polyploidization.

Correlation coefficients for ploidy level and different phenotypic characteristics were between 0.3 and 0.7 for the 500 plants that had been regenerated after mock-transformation. Correlation coefficients higher than 0.5 were found for the features leaf shape, growth on minimam medium, coverage of the protonema with gametophores, and multiple phenotypic deviations from wildtype. We calculated the correlation coefficient for the first 10,000 stable transformands of our mutant collection as well and they were in the same range (0.15-0.7). Correlation lower than 0.5 were calculated for the features growth on full medium, color, and cell shape. Plant structure, coverage with gametophores, leaf shape, growth on minimal medium and multiple deviations were highly correlated (>0.5) with the ploidy level of the mutants.

Although specific phenotypic deviations indicate changes in the ploidy level of plants, identification of polyploid plants (either mutants or regenerants after protoplast isolation) by phenotypic assessment is not possible. Flowcytometry allows unequivocal determination of ploidy level of *Physcomitrella* plants. In our project around 200-350 plants are tested daily.

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P20: The moss *Physcomitrella patens* chloroplast *rpoA* gene is present in the nuclear genome

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Chloroplasts have their own transcriptional apparatus and most chloroplast genes are transcribed by a eubacterial-type plastid-encoded RNA polymerase (PEP). The core subunits of the PEP are encoded by *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* on the chloroplast genome. We have determined the entire nucleotide sequence of the moss *Physcomitrella patens* chloroplast DNA and found absence of *rpoA* in the chloroplast genome. This strongly suggests that chloroplast *rpoA* gene is relocated in the nuclear genome in *P. patens*. Therefore, we initially searched *rpoA* homologs in the *P. patens* EST database (The Physcomitrella EST Programme: <http://www.moss.leeds.ac.uk>) and found an EST clone containing the 3' portion of *rpoA* sequence. Based on the EST sequence, we have carried out 5'-rapid amplification of cDNA ends. The cDNA clone obtained encodes a putative protein of 450 amino acid residues with 45% amino acid identity with tobacco chloroplast-encoded RpoA and 29% identity with *Escherichia coli* counterpart. Therefore, we tentatively designated this protein PpRpoA. PpRpoA contains an N-terminal extension that possibly functions as a chloroplast-targeting signal. To determine the cellular localization of PpRpoA, DNA encoding the first 94 residues containing the putative transit peptide was ligated to the coding sequence of synthetic GFP, and the resultant plasmid was introduced into the *P. patens* protonematal protoplasts. In cells expressing the PpRpoA-GFP fusion protein, green fluorescence was observed in the chloroplasts. This clearly demonstrates that PpRpoA localizes to the chloroplasts. This is the first example of the existence of nuclear-encoded chloroplast RpoA in plants and algae.

P21: A relic of the bacterial peptidoglycan synthesis pathway is retained in moss chloroplasts

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The endosymbiotic theory states that all chloroplasts are derived from a single cyanobacterial ancestor^{1, 2}. It is widely agreed that the chloroplasts of red algae and higher plants have no peptidoglycan layer. Therefore, the evolution from endocytobiont into a wall-less, photosynthetic organelle involved a reduction in and loss of the cyanobacterial cell wall, which is of Gram-negative type.

In the moss *Physcomitrella patens*, treatment with β -lactam antibiotics, which bind penicillin-binding proteins (PBPs) to inhibit peptidoglycan synthesis, results in giant chloroplasts^{3, 4}. Treatment with ampicillin, a β -lactam antibiotic, also led to a decrease in the number of chloroplasts per cell in *Funaria hygrometrica*, and *Polytrichum commune*. These observations suggested that this is a general effect in mosses. Moreover, we showed that antibiotics that inhibited bacterial peptidoglycan synthesis at positions different from β -lactams also inhibited chloroplast division in *P. patens*. D-cycloserine caused a rapid decrease in the number of chloroplasts per cell and its effect was similar to that of β -lactam antibiotics. Fosfomycin affected half of the cells, while vancomycin affected a few cells. Conversely, bacitracin had no effect. Since vancomycin and bacitracin mainly inhibit peptidoglycan synthesis in Gram-positive bacteria⁵, their minimal effects on chloroplast division may depend on differences in peptidoglycan synthesis between the ancestral Gram-negative-type cyanobacteria and Gram-positive bacteria. Fluorescence microscopic images using fluorescent penicillin suggested that PBPs envelope each moss chloroplast in the same way that they surround bacteria. These findings suggest that a relic of the bacterial peptidoglycan synthesis pathway is retained in moss chloroplasts and is involved in their morphology and division.

1. Cavalier-Smith, T. (2000) Trends Plant Sci. 5, 174-182
2. Gray, M. (1992) Int. Rev. Cytol. 141, 233-357;
3. Kasten, B. and Reski, R. (1997) Plant Physiol. 150, 137-140
4. Takano, H. *et al.* Moss2001
5. Bugg, T. D. H. and Walsh, C. T. (1992) Nat. Prod. Rep. 9, 199-215

P22: *FLO/LFY* homologue in *Physcomitrella patens* is necessary for sporophyte formation

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FLORICAULA/LEAFY (FLO/LFY) homologous genes have been isolated from angiosperms, gymnosperms, ferns, fern allies, mosses and liverworts. *Arabidopsis thaliana LFY* positively regulates the expression of some MADS-box genes that decide floral organ identity. This function is conserved in gymnosperm *FLO/LFY* homologues, while the fern, *Ceratopteris richardii FLO/LFY* homologues are not likely to induce MADS-box genes, because the expression patterns of its *FLO/LFY* homologues and MADS-box gene homologues are different. Further approach to the function of its *FLO/LFY* homologues is difficult to make because there is no reliable transformation system. To assess the original function of *FLO/LFY* homologues, *Physcomitrella patens* was used to characterize the genes using its feasible gene targeting technique.

We isolated two *FLO/LFY* homologous genes, *PpLFY1* and *PpLFY2*, from *Physcomitrella patens*. These genes had about 90% identical nucleotide sequences and showed very similar expression patterns. Both *PpLFY1-GUS* and *PpLFY2-GUS* proteins were detected strongly at shoot apices, in young leaves, axillary buds, and archegonia. In the case of expression in archegonium, GUS expression was observed in an entire archegonium at an early stage of development and gradually limited to the venter as it developed.

We obtained six lines of *PpLFY1/PpLFY2* double mutant by retransforming *PpLFY2* single mutant; the *pplfy2* normally developed until sporophytes were formed and had no particular phenotypic difference to the wild type. Double mutants also had no morphological difference from the wild type in protonemata and gametophores and formed normal antheridia and archegonia. However, they never formed sporophytes. Some mature archegonia of the double mutants had a brown plug in the canal, suggesting that fertilization had occurred. Because we could not observe further developed archegonium whose venter enlarged more, we infer that the double mutants arrest development during embryogenesis. Results of further observations including phenotype of *PpLFY1* single mutant will also be reported.

P23: A long standing partnership? – W-boxes and their WRKY companions

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WRKY proteins are a class of transcription factors that are specific to the plant kingdom. Their WRKY domain, a 60 amino acid region, is conserved between all known members. It has been shown that the invariant amino acid sequence WRKYGQK and the C-terminal zinc-finger-like motif are essential for binding to a conserved promoter element, the W Box, with its consensus sequence (T)TGAC(C/T)¹.

WRKY proteins are thought to play an essential role in senescence, wounding, stress and pathogen-triggered signal transduction. Their W-box binding motif was found to be significantly more frequent in a -1.1 kb region upstream of the ATG in the pathogen responsive PR1-regulon of Arabidopsis compared to a non responsive control promoter set². Furthermore, single W-boxes, both in synthetic and natural promoter-reporter gene fusions, were shown to be sufficient to drive specific WRKY dependent expression^{3,4}. Thus WRKY protein W-box interactions were found to be functionally important steps in senescence and stress responses conserved throughout higher plant families.

16 different WRKY gene fragments have been obtained from *Physcomitrella patens* demonstrating that WRKY genes belong to an essential subset of plant genes dating back at least 350 million years in time. We could show that in heterologous expression experiments the W-boxes from Arabidopsis and parsley are functional in *Physcomitrella* protonema.

As genomic sequence information of mosses is sparse, nothing is known so far about W-box frequency, distribution and function. We address the questions whether PpWRKYs also bind to the same motif that is invariant other plants and moreover, if they are involved in the same conserved signal transduction pathways?

1. Eulgem *et al.* (2000): The WRKY superfamily of plant transcription factors. Trends in Plant Science Vol 5, Pages 199-206, May 2000
2. Maleck *et al.* (2000): The transcriptome of Arabidopsis thaliana during systemic acquired resistance. Nat. Genet. 2000 Dec;26(4):403-10.
3. Robotzeck and Somssich (2002): Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev. 2002 May 1;16(9):1139-49.
4. Ruston *et al.* (2002): Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signalling. Plant Cell. 2002 Apr;14(4):749-62.