

Abstracts of Talks

T1: Large-scale functional genomics of *Physcomitrella patens*

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The moss *Physcomitrella patens* is an attractive model system for plant biology and functional genome analysis. It shares many biological features with higher plants but has the unique advantage of an efficient homologous recombination system for its nuclear DNA. This allows precise genetic manipulations and targeted knockouts to study gene function, an approach that due to the very low frequency of targeted recombination events is not routinely possible in any higher plant.

We have developed a high-throughput system for generating a large number (40,000 to date) of *Physcomitrella* mutants via two distinct approaches, based on PEG-mediated transformation of protoplasts. The forward route uses transformation with a gene disruption library produced by transposon mutagenesis of a large number of anonymous cDNA clones; it is expected to yield a saturated *Physcomitrella* mutant collection with insertion mutations in most expressed genes. The reverse route uses knockout DNA constructs specific for defined candidate genes. Quality control with respect to stable integration of the *nptII* marker gene and ploidy level is performed for each transformant. An immediate phenotypic analysis of transformants is made possible by the predominance of the haploid gametophytic state in the life cycle of the moss. Our data show that among the first set of 16,000 transformants, 98% have integrated the *nptII* cassette into the genome. Phenotypic screens revealed that on average 16% of the transformants differed from the wild-type in a variety of developmental, morphological and physiological characteristics, suggesting a highly efficient mutagenesis.

T2: Comparison of the *Physcomitrella* gametophyte transcriptome to the *Arabidopsis* Genome

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The moss, *Physcomitrella patens*, diverged early in land plant evolution from the vascular plant lineage. To understand the change of genomic content during land plant evolution, genome-wide similarity of *Physcomitrella* and *Arabidopsis* was assessed by comparing expressed sequence tag (EST) data of *Physcomitrella* and sequence data of all the protein in *Arabidopsis*. We constructed full-length enriched cDNA libraries from, auxin-treated, cytokinin-treated, and non-treated gametophytes of *Physcomitrella*, and sequenced more than 40,000 clones from both ends. The ESTs are assembled with mRNA sequences of *Physcomitrella* deposited in GenBank, and 23,000 contigs were obtained. Contigs corresponding to the 5' and 3' end of the same clone were paired. Based on these pairs, our clones correspond to 13,000 independent mRNA. We constructed a database of *Physcomitrella* EST, containing the BLASTX search result of every contigs and clones. Currently, 15 Mb of *Physcomitrella* non-redundant EST can be searched with BLAST program in our database.

TBLASTN searches against the *Physcomitrella* database showed that 65% of *Arabidopsis* genes has a homologue in *Physcomitrella* with an *E* value less than or equal to 0.001. Conversely, all the moss contigs (23,000) were used as query in BLASTX searches against nr dataset. Of these, 10,000 showed the highest similarity to a vascular plant gene, and 12,000 found no hit with an *E* value > 0.001 . The remaining 822 contigs had the highest similarity to a gene in an organism other than vascular plants, such as Metazoa and Bacteria, and included genes related to ion transport, DNA damage repair, which may reflect moss specific features. As gametophytes are extremely reduced in Angiosperms, some of the genes functioned in gametophytes may have lost, while most continued to function in sporophytes.

The *Physcomitrella* EST programme (PEP)

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The 3 year BBSRC-funded programme, to sequence *Physcomitrella* ESTs and to offer a service to the plant community for functional analysis of higher plant genes by gene targeting in moss, will complete this year. The achievements of the programme will be discussed; these include linkage of PEP to the NIBB EST collection, resulting in a total of around 60,000 ESTs being available in the public domain; added value to the PEP programme, by construction of a BAC library, by David Lightfoot and colleagues at SIU, Illinois), which is now available for library screening as filter grids (<http://www.moss.leeds.ac.uk>) and progress towards a phenotypic screen service for identifying altered phenotypes.

This talk will draw attention to posters detailing work emanating from PEP resources to be presented at this meeting *i.e.* Lomas *et al.* on bioinformatic analysis (poster P10); Panvisavas *et al.* on analysis of Rho-GTPase genes in *Physcomitrella* (poster P15); Deeks *et al.* on analysis of the *mago-nashi* gene in *Physcomitrella* (poster P3).

The Centre for Plant Sciences at Leeds is one of the largest group of plant scientists in the UK (<http://www.plants.leeds.ac.uk>). Research specialisms at Leeds are allowing a unique combination of expertise to be linked through PEP and applied to fundamental plant functional genomic analysis. We are focussing on tip growth and incorporating approaches looking at roles in development for the cell wall (see talk by Lee *et al.*, T21); intracellular organelles, initially through peroxisomes in collaboration with Dr Alison Baker and extracellular stimuli, initially through light responses involving GATA transcription factors in collaboration with Prof. Phil Gilmartin.

T4: Molecular analysis of tagged *Physcomitrella patens* mutants

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Functional genomics relies on efficient and fast re-isolation of tagged genomic loci. For *Physcomitrella patens* three protocols (iPCR, RAGIL, and Trap-cloning) have been established and optimised for the analysis on integration mutants. iPCR is based on restriction and circular religation of genomic DNA, followed by PCR amplification of DNA regions flanking the marker gene. RAGIL, the Rapid Amplification of Genomic Integration Loci is a derivative of RACE protocols. While iPCR and RAGIL rely on PCR-techniques, Trap-cloning is a plasmid rescue approach to clone and screen for marker gene flanking DNA sequences. All protocols have been tested and applied to targeted knockout mutants of *Physcomitrella* and mutants from our mutant collection and all proved to be applicable for mutant analysis in *Physcomitrella*. Best results have been obtained by Trap-cloning that has been used successfully to re-isolate large flanking sequences (up to 9kb). To date we re-isolated 21 genomic integration loci, homologous and non-homologous. Mutant analysis of tagged mutants is faster than in *Arabidopsis* and in contrast to this model plant a gene/function correlation can be rapidly confirmed in *Physcomitrella* by targeted knockout.

This work is a joint project with BASF Plant Science GmbH.

T5: Efficiency of gene targeting in *Physcomitrella patens*.
I. Factors affecting gene targeting efficiency by direct DNA transfer.

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The high efficiency of somatic homologous recombination in *Physcomitrella patens* is now a widely appreciated phenomenon that is increasingly being exploited as a tool for gene functional analysis. However, the factors affecting the frequency of gene targeting events have not been systematically investigated. These factors include the nature of targeting constructs, the extent of homology between targeted locus and targeting construct, the location and orientation of selectable marker genes within targeting constructs and the means of DNA delivery.

As a part of the EC Framework V 'Pregene' programme, we are systematically analysing these factors. We have selected two members of small multigene families in *P. patens* as targets to determine the conditions made by these factors to the efficiency of gene targeting. These are i) a member of the RNA-binding protein 'Puf-domain' gene family and ii) a member of the gene family encoding the small rho-1-like GTP binding (*rop*) protein.

By introducing an *nptII* selectable marker cassette into different site within genomic clones of these genes, we have created a series of targeting constructs of varying homology length and 3'/5' symmetry. By analysing the nature of the integration events in several hundred stable transformants we are beginning to define, quantitatively, the relationship between homology length, symmetry and targeting efficiency in *P. patens*.

This work is a part of EC framework V, PREGENE project.

T6: 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?

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T7: Evolution of organellar transcription machinery in bryophytes and vascular plants

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Plastids and mitochondria are both considered as descendants of eubacterial endosymbionts. The origin of plastids is thought to be an ancestor of extant cyanobacteria, while the origin of mitochondria is likely to be an ancestor of alpha proteobacteria. This inference is mainly supported by the comparison of genomic sequences of the organelles and various bacteria. However, we recently pointed out that, based on accumulating database information and analytical data of nucleoids, the replication and transcription systems of plastids (or plastid genomic machinery) might not be very similar to those of eubacteria¹. In this communication, we provide further evidence that the genomic machinery of plastids and mitochondria is distinct from that of eubacteria. Two different RNA polymerases (RNAP) are known in plastids of angiosperms. One is encoded by the plastid genome and similar to bacterial RNAP, whereas the other is encoded by the nuclear genome and similar to RNAP of phage T7.

We isolated two cDNAs for the phage-type RNAP (PpRpoT1 and 2) in *Physcomitrella patens*². The protein products of these cDNAs, in the form of fusions with green fluorescent protein (GFP), were both targeted to mitochondria but not to chloroplasts. The transcription activity of isolated chloroplasts was nearly completely inhibited by tagetitoxin, an inhibitor of chloroplast-encoded prokaryotic RNAP. Phylogenetic analysis by the maximum likelihood and neighbour-joining methods clearly indicated that PpRpoT1 and PpRpoT2 form a sister group to the angiosperm RpoT proteins including both chloroplast and mitochondrial isozymes. Results of further analysis indicate that the duplication of RpoT gene leading to the generation of plastid isozyme occurred after the separation of angiosperms. Bryophytes and green algae are, therefore, simple with respect to RNAP: phage-type RNAP in mitochondria and bacteria-type RNAP in chloroplasts. We will also present information concerning DNA polymerases and DNA-compacting proteins^{3,4} in the organellar nucleoids.

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T8: FtsZ proteins in the moss *Physcomitrella patens*

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The bacterial cell division protein FtsZ resembles tubulin in sequence and structure and thus may be the progenitor of this eukaryotic cytoskeletal element. In land plants, several FtsZ proteins are encoded by two small nuclear gene families. Although it has been shown for some of the gene products that they are imported into plastids and play a role in plastid division, the precise function of the products from both gene families is still poorly understood.

The moss *Physcomitrella patens* harbours at least four different nuclear encoded FtsZ homologues. In the following study, we analyzed their genomic structures and evolutionary relationships. For analysis of subcellular localization, we transiently transformed *Physcomitrella* protoplasts with *ftsZ*-GFP-fusion constructs driven by the 35S-promoter. Using confocal laser scanning microscopy, we were able to show different localization patterns for all four fusion proteins.

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T9: *Golden 2-like* genes in *Physcomitrella patens*

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Two maize genes, *Golden 2* and *Golden 2-like (Glk)*, encode putative transcription factors that are thought to be involved in the regulation of chloroplast development. Two *Glk* genes have also been found in rice and *Arabidopsis*, all of which have a conserved DNA binding domain and C-terminal motif. *Glk* genes are included in the GARP gene family, together with *Arabidopsis* response regulators. In *Physcomitrella patens*, two *Glk* genes have been identified (*PpGlk1* and 2). Phylogenetic analysis suggests that these genes form a sister group to the rest of the *Glk* gene family, indicating that independent duplication events have occurred. Both *PpGlk* genes are expressed in the protonema and gametophore, and as in *Arabidopsis*, maize and rice, their expression levels increase on exposure to light. We are currently selecting mutations in both genes to assess whether the *Glk* gene function is conserved between lower plants and higher plants.

T10: Genomic level studies of desiccation-tolerance in *Tortula ruralis* (*Syntrichia ruralis*).

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Much of our understanding of vegetative desiccation-tolerance in plants comes from the study of two model plants that differ extensively in the mechanism by which they achieve this remarkable capability. These models are the dicot *Craterostigma plantagineum* and the widespread moss *Tortula* (or *Syntrichia*) *ruralis*. Our studies have centered upon the bryophyte model that appears to utilize a mechanism for tolerance that includes a constitutive cellular protection component coupled to an inducible cellular repair process. From an evolutionary standpoint this type of mechanism is thought to be the primitive state and as such could provide important clues as to the identity of genes critical to ability to survive severe water deficits. As part of our continuing efforts to understand the mechanisms of vegetative desiccation tolerance we have recently taken a functional genomics approach that includes *Tortula ruralis*. We have established two Subtractive Suppression Hybridization (SSH) libraries for *Tortula* in conjunction with a collection of 10,000 ESTs that are currently under analysis and annotation. A Bacterial Artificial Chromosome (BAC) library is also under construction for physical mapping purposes. Our initial efforts into functional genomics are concentrated on a microarray-based expression analysis, using both the EST collection and the SSH libraries, to establish the identity of those genes that are central to the response. These genes will then form the focus of functional studies in both bryophytes and angiosperm models. The results of our efforts in this area will be presented and discussed. We have also taken a proteomics level approach to unravel the intricacies of a translational control mechanism we have established as critical to the response of *Tortula* to desiccation and rehydration. 2-D gel analysis of polysomal fractions have identified proteins we believe are involved in the formation of messenger ribonucleic acid particles (mRNPs) during a slow desiccation event. These mRNPs selectively sequester rehydrin transcripts and we are interested in the identity, function, and selective binding properties of these proteins. In collaboration with William Hurkman at the ARS Western Region laboratory in Albany California we are attempting to microsequence these proteins in order to expand our abilities to investigate their properties. The results from these studies will also be presented in order to give an overview of our research program involving *Tortula ruralis*.

T11: Cloning and targeted disruption of two genes encoding Snf1-related protein kinases in the moss *Physcomitrella patens*

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The Snf1 protein kinase is a key regulator of the energy and carbon metabolism in fungi. In animals, the homologous AMP activated protein kinase, AMPK, plays a similar role. In plants, there are three subfamilies of Snf1-related kinases. The members of one such subfamily, the SnRK1 kinases, are particularly closely related to Snf1 and AMPK, and are therefore thought to have a similar function. We have cloned two highly similar genes, *PpSNF1a* and *PpSNF1b*, that encode Snf1-related kinases of the SnRK1 type in the moss, *Physcomitrella patens*. Both genes have 9 introns that are perfectly conserved in position, when compared to homologous genes from *Arabidopsis*. They encode proteins of 242 and 245 amino acid residues respectively.

We found that both genes are constitutively expressed at a low level in chloronemal tissue. The *PpSNF1a* and *PpSNF1b* cDNAs can both complement a *snf1* deletion and suppress a *snf4* deletion in yeast. Furthermore both encoded proteins appear to be negatively regulated by their C-terminal domains similar to the yeast Snf1 kinase. Both genes have been deleted using targeted gene disruptions and in the case of *PpSNF1a*, a limited growth phenotype was observed. Construction of a double knock-out mutant strain is in progress and we hope to describe the associated phenotypes if it is viable.

T12: Isolation and characterization of novel aldehyde dehydrogenase (ALDH) genes from the desiccation-tolerant moss *Tortula ruralis*.

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The desiccation-tolerant moss *Tortula ruralis* is an important experimental system for the study of gene control in response to severe water deficit-stress. We have employed EST analysis to discover genes that mediate vegetative desiccation tolerance and have identified two unique members of the aldehyde dehydrogenase (ALDH) gene super family, ALDH7B6 & ALDH21A1. ALDHs represent an important adaptive response to osmotic- and oxidative-stress and are an important pathway for the detoxification of aldehydes by oxidation to their corresponding carboxylic acids. Aldehydes are intermediates in a number of fundamental biochemical pathways that can also be generated in response to a variety of environmental stresses that perturb metabolism such as salinity, cold, heat-shock and desiccation. ALDH7B6 encodes a turgor-responsive ALDH7 protein family homologue with significant similarity to the angiosperm cDNAs 26g, Btg-26 and MF-40. ALDH21A1 encodes a novel ALDH protein. ALDH21A1 is less than 30% identical to known ALDH proteins. Based upon established nomenclature for ALDH proteins, ALDH21A1 describes a novel eukaryotic ALDH protein family designated ALDH21. RNA blot hybridizations were used to analyze expression in response to ABA, UV-C, NaCl and desiccation. ALDH7B6 steady-state mRNA transcript levels are unchanged in response to all treatments and the gene is constitutively expressed. ALDH21A1 steady-state transcript levels increased in response to all treatments and were more abundant within the polysomal mRNA fraction of salt-treated gametophytes. We postulate that ALDH7B6 & ALDH21A1 play an important role in the detoxification of aldehydes generated in response to desiccation-stress, and that ALDH21A1 expression represents a unique stress tolerance mechanism.

T13: Studies of pattern and polarity in Arabidopsis roots

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The Arabidopsis root displays astonishing developmental flexibility despite nearly constant lineage relationships. This organ is therefore particularly well suited to study mechanisms of plant development.

Auxins, with indole-3-acetic acid as the major active form, have diverse roles in plant growth and development that have hitherto been difficult to disentangle. Mutants in auxin transport and response suggest that asymmetric distribution is required for patterning. Redistribution of the auxin concentration peak by laser ablation and by polar auxin transport inhibitors correlates with changes in multiple cell fates and cell- and organ polarity. Thus, auxin and its transport machinery play major roles in organising pattern and polarity in the distal root tip. The role of auxin transport proteins of the PIN and AUX1 families in pattern formation is currently being investigated and a tight link between polarity at the cellular level and pattern formation at the organ level emerges. This link is further substantiated by our studies on the *ORC/SMT1* gene, that links cell polarity to sterol biosynthesis and PIN protein localisation.

The *HOBBIT* gene is required during early embryonic development in the founder cell of the root meristem, and it is expressed throughout development in a cell-cycle dependent manner. The HOBBIT protein likely is a component of the Anaphase Promoting Complex, and we have used the requirement of the *HOBBIT* gene for correct cell fate determination as an opportunity to investigate how cell division and cell fate may be linked in plants, possibly involving the regulation of auxin responsiveness.

Downstream of auxin as a patterning cue, separate distal domains need to be specified in the root tip. The *PLETHORA1*, *PLETHORA2*, *FEZ* and *SOMBRERO* genes are required for proper development of the columella and lateral root cap and current evidence suggests that they act downstream of auxin signaling. The PLETHORA genes encode putative transcription factors.

The *SCARECROW* gene has previously been shown to be required for an asymmetric division that separates endodermis and cortical parenchyma in the ground tissue of the root. Besides this role in radial patterning, we now show that *SCARECROW* is cell-autonomously required in the quiescent center to maintain (in a non-cell-autonomous fashion) the stem cell status of the surrounding initial cells of the root, using a combination of different domain-specific ectopic expression methods. Thus, also this gene contributes to distal specification, and radial and apical-basal patterns appear to be connected in the root tip.

T14: Targeted knockout of genes involved in phytohormone signaling

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The moss *Physcomitrella patens* offers the possibility to investigate the basal mechanisms of phytohormone action, taking advantage of the high degree of similarity to higher plants on the molecular level.

As almost nothing is known about auxin distribution and sensitivity of cells during moss development, we started to characterize it with the help of an auxin inducible reporter gene system¹. Therefore we produced stable transgenic plants with the β -glucuronidase gene (*gus*) driven by the auxin inducible promoter elements GH3 and DR5, respectively. The transgene copy number of several plants was estimated by southern blot analysis. The GUS staining pattern in protonema, buds and gametophores revealed that both auxin inducible elements are functional in *Physcomitrella* but behave differently concerning staining intensity and response time. The staining was predominantly visible in buds and stems of gametophores. Characterized plants will be used as platforms for knock-outs of genes encoding proteins involved in auxin transport like the auxin efflux carrier PIN. We found an EST representing a putative *Physcomitrella pin* homologue. A molecular analysis of putative knock-out plants is carried out at the moment.

In addition, we isolated two different *gh3* homologues expressed in *Physcomitrella* protonema (PpGH3 1 and PpGH3 2). A possible third homologue is existing as an EST found in a gametophore specific library. We knocked out PpGH3 1 and the phenotype is currently under investigation.

One of the key cell-cycle regulatory points is the G1/S transition controlled by the so called Rb pathway. In this pathway expression of *cycD* and *cdkA*, that build a Rb phosphorylating kinase in a binary complex, is controlled by cytokinin and auxin, respectively² and by sugar availability. We isolated the *Physcomitrella* homologues for the essential members of this pathway. This is the first time that all important members of this pathway have been found in such an evolutionary old organism. We proved the G1/S regulating function of the *Physcomitrella* *CycD* homologue by complementation of a yeast mutant, which is deficient for G1/S cyclin genes and knocked out this gene in *Physcomitrella*. The knockout mutants showed accelerated caulonema development and enhanced and quicker formation of buds in the presence of cytokinin.

Furthermore, knockout mutants for the Rb homologue have been created, which are currently under investigation.

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T15: Isolation and knockout of *Physcomitrella patens* histidine kinase genes homologous to *AtCre1*

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Recent studies have demonstrated that cytokinin signaling involves a multistep two-component signaling pathway and that hybrid histidine protein kinases (AHKs/CRE) serve as cytokinin receptors in *Arabidopsis thaliana* (Inoue *et al.*, Nature 2001). In *P. patens*, cytokinins control the critical developmental stage of bud formation on protonema filaments and development to the leafy gametophores. These properties and the ease of gene replacement make *P. patens* a highly attractive model to study cytokinin mechanism of action, at the cellular and molecular level, and prompted us to look for *AtCre1* homologous genes. Using degenerate oligonucleotides designed from *AHK4* and other *AHK* plant homologs, we identified two *P. patens* *AHK* homologs (*PpCRE1* and *PpCRE2*) and full length cDNAs have been cloned. We obtained knockout transformants for these genes using corresponding genomic DNA fragments, interrupted by a positive selection marker. The molecular characterization of the knockout plants will be presented and their developmental phenotype in response to cytokinin will be discussed.

T16: Polar auxin transport is integrated in diploid generation of sporophytic tissue but not in haploid gametophytic shoots in mosses

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The control of auxin distribution, regulated by synthesis, metabolism and polar auxin transport (PAT), is important for various physiological phenomena and morphogenesis of shoots in diploid generation of angiosperms. There are, however, few literatures about the regulation of auxin distribution in mosses. Mosses develop a shoot-like structure, the gametophore in the haploid generation, although this shoot-like structure evolved independently of the diploid shoots of angiosperms. In their diploid generation, mosses generate a rather simple structure, the sporophyte, which does not develop lateral organs. It is intriguing to discover how the distribution of auxin is regulated and how it controls physiology and morphogenesis in the development of the gametophore and sporophyte of mosses.

To reveal the distribution of auxin and responses to it in mosses, we fused the promoter of auxin-inducible gene, *GH3* from soybean to *GUS* reporter gene and introduced it into *PpMADS2* locus to create *GH3:GUS* transgenics of *Physcomitrella patens*. We examined the expression pattern of the *GUS* reporter gene in the transgenic plants. We also tested PAT directly by using conventional agar-block methods with ¹⁴C-IAA in gametophores and sporophytes in some mosses.

Neither the polar movement of ¹⁴C-IAA nor the effect of auxin transport inhibitors was detected in gametophores. In contrast, PAT was detected in sporophytes, which was suppressed by auxin transport inhibitors, as seen in angiosperms. Drastic change was observed in *GUS* staining pattern during embryogenesis of sporophytes, and the treatment of auxin transport inhibitor caused abnormal development of embryos. These results suggest that PAT plays significant roles in embryogenesis of the sporophyte generation, whereas it does not appear to play a role for the development of gametophores in mosses.

Hartman talk

T 18: The Arp2/3 complex in *Physcomitrella patens*: possible role in the gravitropic response

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The Arp 2/3 complex is ubiquitous in non plant eukaryotes (see Mullins and Pollard,1999) and plays a crucial role in two major properties of the actin network: (1) it favors the polarization of F-actin by capping the barbed end of the actin filament, and (2) it permits branching of actin filaments by binding the pointed end of a filament to the lateral side of another filament. The complex is composed of seven subunits (Arp2, Arp3 and ARPC1 through 5 - see May, 2001), all of which are required for the two functions but the role of each subunit is not fully understood. In plants, no functional evidence for the presence of this complex has been described. However, analysis of different cDNA and gDNA sequences available in databases confirms the presence of all subunits in *Arabidopsis* and other plants.

Physcomitrella patens caulonema cells show a negative gravitropic response in the dark. Moreover, as in other mosses, the response is confined to the tip growing cells. Organisms respond to the gravity vector by a mechanism involving two main components: a "sensor" or statolith which could be a subcellular organelle, and, a network of actin and/or microtubules which play a role of integrator of the signal and triggers subsequent polar tip growth (Braun, 1997). Although a precise role of the actin cytoskeleton in this polar response remains unclear, the regulatory function of the Arp2/3 complex could be a key factor in the understanding of polar tip growth in response to gravity.

In this presentation, we will focus our interest on the subunit Pp-p20 (ARPC4) of the Arp2/3 complex in *P. patens*. Using RT PCR approach, a cDNA coding for a p20 has been isolated. It codes for a 169 amino acid protein with a molecular weight of about 20kDa. The amino acid sequence of Pp-p20 is 66% identical and 85% similar to the human p21 homologue and appears to be represented in the *P. patens* genome as a single copy gene, as determined by Southern analysis.

Protoplasts transformation has been performed with a construct containing Pp-p20 cDNA containing a triple HA epitope tag at the 5' end. Stable lines of *P. patens* containing this construct were analyzed for the accumulation of the protein. Lines positive for the presence of the PP-p20 protein were then tested for their response to gravity. We will report on the altered gravitropic response in some of these lines and discuss the possible role of the Arp2/3 complex in the gravitropic response in *P. patens*.

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T19: The morphogenetic gradient determining axis polarity in regenerating protoplasts of the moss, *Ceratodon purpureus*, involves phytochrome.

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Ceratodon protoplasts regenerate by polar outgrowth, forming cell filaments directly. In red light, outgrowth is polarised by the direction of the light source. We have shown, by the re-orientation of protoplasts regenerating in uni-directional red light, that development of the regeneration axis occurs in two steps (Cove D.J., Quatrano, R.S & Hartmann, E. 1996, Development 122, 371). There is a lag of about 9 hours following reorientation, before the regeneration axis becomes aligned to the new light direction. The orientation of the aligned axis, *i.e.* whether outgrowth occurs towards or away from the light source, is determined more slowly, so that the regeneration axes of protoplasts aligning to a new light direction following reorientation, show little or no tendency to orient their polar outgrowth positively.

To investigate a possible role for phytochrome in the response to light during polar axis formation, we studied the effect of treating protoplasts which had started to regenerate in red light, with far-red light before they were re-oriented and exposed to red light from a new direction. The far-red treatment results in the "memory" of the first light direction being lost more quickly, and a more rapid response to the new light direction, compared to the control (no far-red) treatment. However, if far-red light treated protoplasts are briefly returned to red light from the original direction before reorientation, the protoplasts' normal regeneration programme is restored, *i.e.* protoplasts treated in this way resemble control protoplasts.

We propose that the morphogen gradient that establishes the polarity of the protoplast regeneration axis, must be stabilised by phytochrome in its P_{FR} form, resulting in the response to a new light direction being slow, but that the gradient is less stable when phytochrome is in its P_R form, allowing a response to a new light direction to occur more rapidly.

T20: Identification of two genes encoding a ubiquitin-like protein, which is predominantly expressed in apical cells of the moss *Physcomitrella patens*.

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Postembryonic growth of land plants occurs from the meristem, a localized region that gives rise to all adult structures. Meristems control the continuous development of plant organs by balancing the maintenance and proliferation of stem cells, and directing their differentiation. Mosses have two types of meristems: a protonema apical cell and a gametophore apical cell. The apical cell is a single meristematic cell that is maintained through self-renewal, and gives rise to various organs. In the moss *Physcomitrella patens*, the developmental process of the apical cell is well defined at the cellular level, and gene targeting based on homologous recombination is feasible. Thus, apical cell differentiation in *P. patens* is used as a model system for studies of meristem development in land plants. To understand molecular mechanism of formation and maintenance of the apical cell, we have generated gene-trap/enhancer-trap lines to identify genes specifically expressed on the apical cell (Hiwatashi et al. 2001). Here we reported an isolation of two ubiquitin-like genes, *yh78* gene from a gene-trap line YH78 and a *yh78*-related gene, *pph27a22*, found in the *Physcomitrella* EST database. Both *yh78* (606 residues) and *pph27a22* protein (612 residues) contain two tandem repeats of a ubiquitin-like domain close to their N-terminals. The translation fusion protein with GUS reporter protein was accumulated preferentially in apical cells of both protonemata and gametophores, suggesting that these genes function in the apical cells. We carried out the disruption of *yh78* and *pph27a22* using the gene-targeting technique. Disruption of *yh78* or *pph27a22* alone did not result in any noteworthy phenotype, but double disruption of *yh78* and *pph27a22* exhibited abnormal apical cells and altered branching pattern in protonemata. The growth of protonemata of double disruptant was slower than that of wild type. Thus *yh78* and *pph27a22* genes are functionally redundant and are involved in formation and/or maintenance of the protonema apical cell. Phenotype of the gametophore apical cell of the double disruptant is under observation.

Hiwatashi, Y., Nishiyama, T., Fujita, T., and Hasebe, M. (2001). Establishment of gene- and enhancer-trap systems in the moss *Physcomitrella patens*. *Plant J.* 28: 105-116.

T21: Arabinogalactan proteins and protonemal growth of *Physcomitrella patens*

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The single-cell filamentous growth of moss allows cell wall molecules to be accessed directly at cell surfaces, providing an opportunity to analyse cell wall components without the need for the invasive disruption of multicellular tissue. The analysis of the public database of *Physcomitrella* ESTs has highlighted several ESTs with strong homology to anthophyta AGPs. We have directly examined the surface of moss cells using monoclonal antibody probes in a novel whole mount fluorescent-labelling technique. The development of protonemal tissue has been studied from both the regeneration of protoplasts and the germination of spores. The LM6 (1→5)- α -L-arabinan epitope has been found associated with the plasma membrane. This is a novel observation, suggesting the presence of a proteoglycan such as an arabinogalactan protein (AGP). AGPs are implicated in plant growth and development and specifically interact with a group of synthetic phenazo dyes known collectively as the Yariv reagents. Treatment of protonemal tissue with β -glucosyl Yariv reagent resulted in reversible inhibition of growth - implicating AGPs in apical extension. We have used β -glucosyl Yariv reagent to purify AGP(s) from *Physcomitrella* and further characterisation is in progress.

T22: Efficient gene substitution in the moss *Ceratodon purpureus*

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In the moss *Physcomitrella patens*, homologous recombination is a powerful tool for gene targeting. However, *Physcomitrella* is the only moss species for which efficient homologous recombination has been demonstrated. Here we show that gene targeting is possible also for the moss *Ceratodon purpureus*, a species often used as model organism for phototropism and gravitropism. For gene replacement studies, we chose the *Ceratodon* mutant *ptr116*. The phenotype of this mutant is caused by a single point mutation that results in a stop codon within the coding region of the heme oxygenase gene. As a result, the biosynthesis of the phytochrome chromophore is blocked in this mutant. Therefore, the phytochrome-controlled phototropic response is inhibited and the chlorophyll level is down-regulated in *ptr116*. The mutant was transformed with the wild-type heme oxygenase gene in which two silent mutations had been introduced to generate new nuclease restriction sites. After transformation, lines with a rescued phenotype were found at a rather high frequency. Restriction digest of PCR products indicated that in 75% of analysed lines the rescue resulted from gene replacement. This was confirmed for selected transformants by Southern blot. These results show that efficient gene substitution *via* homologous recombination in *Ceratodon* is possible.

T23: Arp3 is involved in plant cell elongation mediated by rearrangement of actin microfilaments.

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Cellular polarity is a cell feature in all eucaryote cells that results in the asymmetrical distribution of cell compounds and organelles influencing growth and cell division. Extracellular or intracellular signals can be propagated by pathways mediated by small Rho-GTPases leading to final rearrangement of cytoskeleton structures.

Three different types of linear proteinaceous polymers make the cytoskeleton: actin filaments, microtubules and intermediate filaments. In plants, research has focused on the actin and the microtubule network, but there is still little known about regulation of architecture and dynamics of actin cytoskeleton. Comparisons of yeasts, moulds and animal cells have revealed existence of several actin-related proteins (ARPs) that share 20-60% sequence identities with conventional actins. Arp2 and Arp3 are part of a seven-subunit protein complex that choreographs the formation of branched actin network, binds profilin, and nucleates and polymerises F-actin.

We have isolated *arp3* gene from a lambda-FIX-II genomic library (Leeds) of *Physcomitrella patens* by heterologous screening using the Arabidopsis *arp3* genomic sequence as a probe. The length of the *Pparp3* genomic sequence is 2811 bp and contains nine exons that encode a predicted 417 aminoacid peptide that shows a high degree of conservation compared to Arp3 from other eucaryotes. Disruption of the *Pparp3* gene is performed by deletion of 900 bp including exons 4, 5, 6 and partially exon 7 and replacing it by 35S hygromycin phosphotransferase flanked by P-lox sites. This *arp3-KO* vector was employed to transform the protoplasts. 72 independent hygromycin resistant colonies displayed a marked morphological specificity. Chloronemal cells were short with a length/width ratio of almost 1:1 compared to a wild type ratio of 7:1. The caulonema phase is completely absent and auxin treatment did not induce the reappearance of this cell type. Buds formed directly on chloronemal cells and developed into gametophores. Gametophores are shorter than wild type ones but leaf morphology remains unchanged. During gravitropic experiments in darkness young gametophores display the typical etiolation response.

In order to investigate subcellular structure in the knockout lines we retransformed a GFP-Tn-expressing strain with the *arp3-KO* vector bearing neomycin-phosphotransferase. GFP-Tn binds to F-actin and brightly labels a cortical branched network of actin cables that are aligned parallel to the axis of growth as are the cortical star-like structures connected with them. In the *arp3-KO* strains the actin network is disorganised and completely lacks actin bundles and star-like structures. Further detailed analysis will be provided using confocal microscopy.

This is the first report of an Arp3 knockout in any multicellular organism with a viable phenotype. Arp2/3 complex as part of cytoskeleton machinery appears to be important for plant cell elongation processes. The role of Arp3 also seems to be crucial in the execution of signalling induced by auxin. Point mutation approach in combination with two-hybrid yeast system could provide further information about plant actin cytoskeleton structure and dynamics.