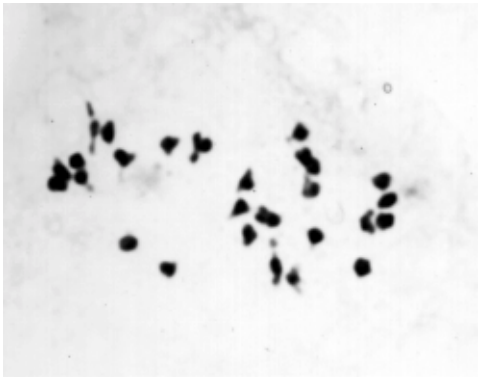
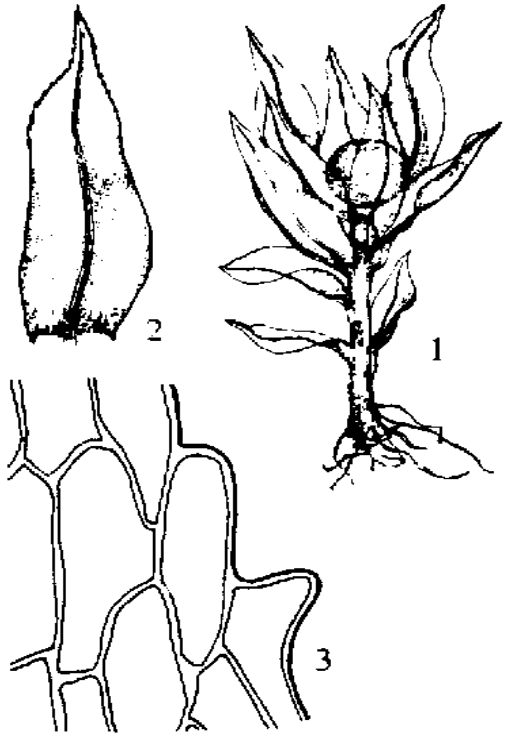
Moss 2000:
a meeting dedicated to moss biology

June 30-July 2 2000

Villars s/Ollon - Switzerland



by courtesy of Martha Newton



Organizers: Jean-Pierre Zrÿd and Didier Schaefer

This meeting is dedicated to the memory of the late

Patricia Geissler

bryologist

Villars s/Ollon – Switzerland
Eurotel -Villars

Program**: June 30th to July 2nd 2000**:

Friday 30 June

14h00	Jean-Pierre Zrÿd Université de Lausanne, Switzerland
	Welcome address
14h15	Andrew C. Cuming, Leeds University, UK
	Progress on PEP: *Physcomitrella* for functional genomics
15h00	Didier Schaefer, Susan Vlach, Mikhail Chakhparonian, Jean-Pierre Zrÿd, (Université de Lausanne, Lausanne, Switzerland), Klaus von Schwartzenberg, Nicole Houba-Hérin, C.Pethe and Michel Laloue (INRA Versailles, France)
	Homologous recombination and functional genomics in Physcomitrella patens: how far can we go ?
15h45	Coffee Break
16h15	Andrew J. Wood, Melvin J. Olivier, Xinbo Chen, Youngoo Cho and Qin Zeng Southern Illinois University, US
	***Tortula ruralis*, Desiccation-tolerance & ESTs: What have we learned from Structural Genomics?**
17h45	Poster session
19h00	Dinner

Saturday 1 July

9h00	K. Hentschel, T. Münster, H. Saedler and G. Theissen, Max-Planck-Institut für Züchtungsforschung Abteilung für molekular Planzengenetik, Köln, Germany
	Defining Mads-box gene function in the moss *Physcomitrella patens*
9h45	Coffee break
10h15	Rumiko Kofuji and Mitsuyasu Hasebe, National Institute for Basic Biology, Japan
	Expression patterns of floral homeotic gene homologues in the moss *Physcomitrella patens*, and its application to the evolution of reproductive organs in lands plants

11h00 Nathinee Panvisavas, Celia Knight, Andrew C. Cuming, David Cove (Leeds University, UK) and Ralph Quatrano (University of Washington, St Louis, US)

Gene targeting of a rop/rac homologue in *Physcomitrella patens*

12h00 Lunch

14h00 Patricia A. Carneiro and Ralph Quatrano University of Washington, St Louis, US

MOSS: A genetic system to study cellular polarity

14h45 Ori Schipper, ETH Zürich, Switzerland

Role of expensin(s) in the development of *P. patens*

15h30 Coffee break

16h00 Tilman Lamparter, Gerhard Bruecker, Franz Mittmann, Mathias Zeidler, Alexander Repp, Elmar Hartmann and Jon Hughes (Freie-Universität, Berlin, Germany).

A-phototropic mutants of the moss *Ceratodon purpureus*

17h30 Poster session

19h00 Dinner

Sunday 2 July

Morning (departure around 9h00) :

Excursion to Pont-de-Nant, the alpine botanical Garden of the University of Lausanne and a natural flora and game reserve.

12h00 Pic-nic

Afternoon (departure around 13h30):

Excursion in the alpine rain-forest of Solalex and if weather permit to Anzeindaz (good mountain shoes, warm shirt and rain coat recommended)

19h30 Dinner

NB: On Friday evening, an administrative and prospective session is scheduled (international cooperation and projects, next MOSS meeting, etc.) –

Moss 2000 - Posters

1. **PEP: The *Physcomitrella* EST Programme**. Celia Knight, Andrew Cuming, David Cove, David Westhead and Ralph Quatrano

2. **Cytokinin metabolism in *ove* mutants of *Physcomitrella***. P. Schulz, A. Hofmann, E. Russo, M. Laloue, K. v. Schwartzenberg

3. **Full-length cDNA activation system in *Physcomitrella patens***. Tomomichi Fujita, Yuji Hiwatashi, Tomoaki Nishiyama, Keiko Sakakibara, Mitsuyasu Hasebe

4. **Characterization of Homeobox Genes in the Moss, *Physcomitrella patens*** Keiko Sakakibara, Tomoaki Nishiyama, Mitsuyasu Hasebe

5. **Tagged Mutagenesis and Gene-trap in the Moss, *Physcomitrella patens* by Shuttle Mutagenesis.** Tomoaki Nishiyama, Yuji Hiwatashi, Keiko Sakakibara, Masahiro Kato, Mitsuyasu Hasebe

6. **Generation of gene/enhancer-trap lines in the moss, *Physcomitrella patens* and expression patterns of a reporter gene (GUS) in gene/enhancer-trap lines**. Yuji Hiwatashi, Tomoaki Nishiyama, Mitsuyasu Hasebe

7. **Stable chloroplast transformation of the moss *Physcomitrella patens* using GFP as reporter**. Jeong-Sheop Shin

8. **Effects of low frequencies magnetic field on the moss *Physcomitrella patens***. Julia Proussakova, Pierre Zweiacker, Michel Ianoz and Jean-Pierre Zrÿd

9. ***In vivo* observation of the actin network in *Physcomitrella patens***. Andrija Finka, Didier Schaefer, Pierre-François Perroud. Jean-Pierre Zrÿd

10. **Using fission yeast to identify and study plant cell polarity genes**. Pierre-François. Perroud, Didier Schaefer and Andrija Finka

11. **New tools for functional genomics in *Physcomitrella patens***. Mikhail Chakhparonian, Didier Schaefer and Jean-Pierre Zrÿd

12. **Dynamic of the cytoskeleton during the polarotropic orientation of the first division of *P. patens* protoplasts** Alexander Skripnikov, Didier Schaefer and Jean-Pierre Zrÿd

13. **Evolution of plastid division.** Justine Kiessling, Sven Kruse, Ralf Reski, Eva Decker

14. **Use of *Physcomitrella patens* to study the funktion of the plant specific WRKY transcription factor family** Dierk Wanke, Patrick Giavalisco, Franziska Turk, Didier G. Schaefer, Paul J. Rushton and Imre E. Somssich

15. **Development of the moss *Ceratodon purpureus* as a model for plant biotechnology.** Mary Honma

LES DIABLERETS, June 29-30th , 2000 - Hôtel le Chamois

Satellite symposium

Symposium du IIIeme cycle romand en sciences biologiques – Biologie Végétale

Homologous recombination and functional genomics in plants

Organisers : Didier Schaefer and Jean-Pierre Zrÿd

Speakers and topics (the symposium start at 14h00 on Thursday 29)

Dr. Neal SUGAWARA

Rosenstiel Basic Medical Sciences Research centre, Brandeis University, Waltham, USA

"Genetic requirements for break-induced recombination in S. *cerevisiae*"

Pr. Jürg KOHLI
Institute of General Microbiology, University of Bern, Switzerland
"Meiosis and recombination hotspots in fission yeast"

Dr. Paul BUNDOCK
Institute of Plant Sciences, Leiden University, The Netherlands
"Homologous recombination and *Agrobacterium tumefaciens*"

Dr. Ivan MATIC
Faculté de Médecine Necker, Université de Paris V, France
"Homologous recombination, horizontal gene transfer and bacterial evolution"

Dr. Joska ZAKANY
Dept de zoologie, Sciences III, UNI Genève, Switzerland
" Homologous recombination and functional genomics in mouse ES cells"

Dr. Roman ULM
Friedrich Miescher Institute, Basel, Switzerland.
"Genotoxic stress and homologous recombination in *Arabidopsis thaliana*"

Dr. Didier SCHAEFER
Institute of Ecology, Plant Cell Genetics, UNILausanne
"Homologous recombination and functional genomics in *Physcomitrella patens*"

Oral presentations

Progress on PEP: *Physcomitrella* phor phunctional genomics

Andrew C. Cuming,
Leeds University, UK

PEP is the *Physcomitrella* EST programme: a resource for functional genomics supported by the UK Biotechnology and Biological Sciences Research Council being developed in a collaboration between Leeds University in the UK and Washington University in the US. The programme aims to deposit *ca.* 30,000 partial cDNA sequences derived from *Physcomitrella patens* in public access databases as a resource for the world plant science community to utilise the capability of *Physcomitrella* in undertaking homologous recombination-mediated gene modification. To date, approximately 10,000 sequences have been deposited in GenBank, and are also accessible through the PEP website. Registration on this site permits PEP users to conduct BLAST searches of the PEP sequence database, in order to identify *Physcomitrella* homologues of genes previously identified in other organisms, and to order individual clones, cDNA and genomic libraries, and vectors designed for the construction of gene knockout or gene replacement vectors.

Currently available PEP resources include cDNA libraries constructed from protonemal tissue of both *Physcomitrella patens* and *Ceratodon purpureus*, and genomic libraries from both organisms. Additionally, plasmid vectors containing an *nptII* cassette flanked by a multiple cloning site on either one or both sides of the antibiotic resistance gene are available for the construction of allele replacement and gene disruption vectors. We are now evaluating the use of an *in vitro* transposition system for the creation of rapid gene disruptions in sequences which lack conveniently situated restriction sites, and the potential of this system will be discussed. Finally, we are providing training and support for laboratories with no previous experience of *Physcomitrella* biology, in the form of annual workshops in *Physcomitrella* methodology, and a transformation service on a part-cost basis.

Homologous recombination and functional genomics in *Physcomitrella patens* : lessons from the adenine phosphoribosyl transferase locus.

Schaefer Didier, Vlach Susan, Chakhparonian Micha and Zrÿd Jean-Pierre.
Institut d'Ecologie, Lab. de Phytogénétique Cellulaire, BB, UNI Lausanne

Klaus von Schwartzenberg, Nicole Houba-Hérin, Claude Pethe and Michel Laloue:
Laboratoire de Biologie Cellulaire, INRA, Versailles.

Fine analysis of gene function is based on the ability to generate specific mutations in the genome by homologous recombination mediated transformation. *Physcomitrella patens* is the only plant so far where this type of approach is possible, making it an extremely valuable model system to study plant biology. To characterise gene disruption and allele replacement events in *Physcomitrella,* we have chosen the adenine phosphoribosyl transferase gene (apt), taking advantage of the fact that its loss of function generates plants resistant to the adenine analogue 2,6-diaminopurine (DAP). Both cDNA and genomic sequences of the moss *apt* gene were isolated, characterised, and used to build the replacement vectors used in this study. Pp*apt1* gene is a single copy gene composed of 7 exons ranging in size from 58 to 317 bp and the cDNA is ca 920 bp.

Protoplasts were transformed with a linear replacement cassette carrying a 35S-neo marker inserted in the cDNA and kanamycin resistant strains were recovered at a frequency of 5 in 100000 regenerants. Out of 100 kanamycin resistant strains, targeted integration in the *apt* genomic sequences occurred in 10 of them and gene conversion was successfully achieved in 2 out of 10 targeted transformants. Molecular and sequence analyses revealed that conversion was achieved by 2 homologous recombination events occurring within sequences of continuous homology as short as 52 and 187 bp. In the other clones, targeted integration was achieved by the insertion of the transforming DNA by a single homologous recombination event occurring within the longer stretch of continuous homology (187 or 311 bp of exon VII) associated with an end-joining reaction between vector and genomic sequences. Our data also indicate that the presence of short stretches of heterologous sequences at the extremities of the transforming DNA, in this case the Not I-Eco RI linkers flanking the *apt* cDNA, impair the efficiency of replacement events. Finally, nine out of ten clones were carrying multiple direct repeats of the transforming DNA integrated in the *apt* locus, and sequence analyses of internal junctions revealed that the junctions were identical between the repeats. Interestingly, both loss of transforming DNA sequences, or gain of genomic DNA sequences were observed in the internal junctions. This observation and the fact that multiple repeats are always in direct orientation suggests that homologous recombination events are achieved in a DNA synthesis-dependent process.

Moss protoplasts were also transformed with 3 different genomic fragments (1.2, 4.0 and 5.6 kb) carrying a deletion in the *apt* genomic sequence (150 or 1100 bp). Transformed strains were selected on DAP supplemented medium and DAP resistant *apt-* strains were recovered at frequencies as high as 1 in 1000 regenerants. Double strand breaks in the transforming DNA increased transformation frequencies up to 100 fold as compared to circular DNA and maximal transformation frequencies

were already observed with as little as 500 and 600 bp of sequence homology flanking a 150 bp deletion. Out of 50 DAP^R strains analysed, gene conversion resulting in the deletion of part of the *apt* genomic sequence without integration of any other vector sequences was observed in 25 to 65% of the clones depending on the transforming vector. Multi-copy replacement and targeted insertion mediated by a single homologous recombination event occurring within the 5' or the 3' homologous sequence associated with and end-joining reaction was observed in most other DAP resistant clones, as observed with cDNA based replacement vectors. Interestingly, double cross-over mediated gene replacement and single cross-over mediated insertions occurred at similar frequencies.

Unexpectedly, in addition to DAP resistance, *apt-* mutants of *Physcomitrella* displayed a strong developmental phenotype characterised by abortive bud development. Leafy shoots do not grow beyond the third to fourth whorl of leaves and these do not develop fully on minimal medium. The phenotype is even stronger on NH_4^+ supplemented medium where buds develop into microcalli without visible differentiated leaves. Furthermore, it was found that *apt–* strains were hypersensitive to exogenously supplied adenine and died within 3-4 weeks when grown on minimal medium supplemented with 0.25 to 0.5 mM adenine. At the biochemical level, feeding experiments with radiolabelled adenine revealed that *apt-* strains were no more able to convert adenine into adenylates and this was confirmed *in vitro* on moss extracts. We therefore conclude that the observed phenotype of *apt-* strains of *Physcomitrella* is probably associated with the accumulation of adenine in the plants resulting from their inability to recycle adenine into AMP.

The general use of *Physcomitrella* as a model system for plant functional genomics is also dependent on the ability to complement mutations in moss with higher plant genes. The strong developmental phenotype of *apt-* strains provided us with an ideal situation to test if *Arabidopsis apt* genes can complement the mutation in *Physcomitrella*. The *apt-* strain Xeh6 that carries a 150 bp deletion in Pp*apt1* was transformed with the *apt-1* or the *apt-2* gene of *Arabidopsis* cloned under the control of the rice actin1 gene promoter. Both genes complement the *apt-* phenotype observed in moss, and biochemical and molecular analyses of these strains confirmed that complementation was correlated with the expression of the *Arabidopsis apt* gene. These results demonstrates that *Arabidopsis* genes are functional in *Physcomitrella* and that the functional information obtained in moss is relevant to higher plant biology.

Fine functional genomics requires the possibility to generate specific mutations in a gene and this can only be achieved by gene conversion events. Our work on Pp*apt1* demonstrate that such approach is straightforward in *Physcomitrella*, with frequencies, sequence homology requirement and patterns of integration similar to those observed in budding and fission yeast. This situation is unique in plants. This work also describes a rather unexpected result: i.e. that loss of function of the *apt* gene leads to an accumulation of adenine and alters plant development: the involvement of adenine in the control of higher plant development is currently tested in Arabidopsis *apt* mutants. Finally our results demonstrate that the corresponding genes from *Arabidopsis* can functionally complement the mutation, an essential requirement for the general use of *Physcomitrella* as a model system to study plant biology.

Thus *Physcomitrella patens* provides a outstanding and so far unique model system to study plant biology with the powerful functional genomic approaches applied in yeast.

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Tortula ruralis, Desiccation-tolerance & ESTs: What Have We Learned From Structural Genomics?

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*T*ortula ruralis is an important model system for the study of plant vegetative desiccation-tolerance and post-transcriptional gene control. Gametophytes can be dried to extremely low relative water contents and the gametophytes fully recover normal activity upon rehydration. We have characterized more than 160 ESTs from a cDNA expression library derived from the polysomal, mRNP fraction of desiccated gametophytes. We have established one of a limited number of moss EST databases (see Table 1) and are using it to define cis-acting elements that control the formation of mRNA 3'-ends and analyze gene expression during desiccation & rehydration. Using traditional RNA blot analysis were are fully characterizing numerous cDNAs of interest including an early light-inducible protein (ELIP), an AAA (ATPases Associated with various cellular Activities) super-family homologue, several ribosomal proteins, and a small number of genes with no significant similarity to previously characterized genes. These studies will be presented and discussed in relation to altered mRNA stability and proposed mechanisms of desiccation-tolerance. Supported, in part, by USDA grant # 9735100 to AJW. *presenting author; wood@plant.siu.edu

Table 1. Inventory of *Tortula ruralis* ESTs with significant similarity to genes from other organisms.

Clone	Accession #	Putative Identity/Homolog	Clone	Accession #	Putative Identity/Homolog
RNP1	AI305036	HSC70 BIP mRNA	RNP24	AI305059	Nucleotide-binding protein
RNP4	AI305038	Ribosomal protein L15	RNP27	AI305062	Ribosomal protein L19
RNP10	AI305045	V-type ATPase	RNP29[a]	AI305064	Desiccation-related protein
RNP11	AI305046	NADH-oxidoreductase	RNP33	AI305068	High MW antigen
RNP20	AI305056	Ribosomal protein S14	RNP44	AI305079	Glutathione-S-transferase
RNP35	AI305070	NAD-dependent fdh	RNP55	AI305090	Extensin
RNP37	AI305072	Ribosomal protein S16	RNP57	AI305092	ERD10

(dehydrin)

RNP47	AI305082	Ribosomal protein L23	RNP63	AI305098	Phenylalanine hydroxylase
RNP49	AI305084	Histone H3 protein	RNP73	AI305068	Catalase
RNP51	AI305086	Chlorophyll a/b apoprotein	RNP74	AI305069	RHD3
RNP54	AI305089	Rieske iron-sulfur protein	RNP78	AI304973	Core Protein
RNP59	AI305094	S-phase-specific protein	RNP80[a]	AI304975	Desiccation-stress protein
RNP66	AI305101	Ribosomal protein L22	RNP83	AI304978	Aminopeptidase
RNP68	AI305103	Lipoxygenase	RNP94	AI304989	HVA-1
RNP70	AI305105	*psaG* gene, PSI	RNP124	AI305019	Group 3 LEA-like
RNP71	AI305016	Chlorophyll-binding protein			
RNP72	AI304967	Orthophosphate dikinase			
RNP82[a]	AI304977	10kDa polypeptide, PSII			
RNP86	AI304981	Cinnamic acid hydroxylase			
RNP114	AI305009	Ribosomal protein L30			
RNP119	AI305014	Group 3 LEA			
RNP123	AI305018	GapD homologue			
RNP128	AI305023	Ribosomal protein S8			
RNP130	AI305025	ADP-gluc phosophorylase			
RNP155[b]	U40818	RAB 24			
RNP213[c]	AI313704	Polyubiquitin			

The putative functional identity of each gene was established by sequencing > 375 bp of the 3' end of each cDNA. Genes presented exhibited significant similarity at the nucleotide (left column) or amino acid level (right column). [a]two copies of this clone have been identified; [b]also known as Tr155, [c]also known as Tr213.

Defining MADS-box gene function in the moss *Physcomitrella patens*

Katrin Henschel, Thomas Münster, Heinz Saedler and Günter Theißen: Max-Planck-Institut für Züchtungsforschung, Abteilung für molekulare Pflanzengenetik, Carl-von-Linné-Weg 10 D 50829 Köln 0049/0221/5062-121

MADS-box genes encode a family of highly conserved transcription factors which play important roles in signal transduction and developmental control in plants, animals and fungi. In angiosperms MADS-box genes are components of complex networks of genes which e.g. sculpt the structure of flowers. In gymnosperms MADS-box genes are also involved in the development of reproductive structures.

MADS-domain proteins, like many other eukaryotic transcription factors, have a modular structural organization. This structure, including MADS (M-), intervening (I-), keratin-like (K-) and C-terminal (C-) domains, is very similar in almost all known seed plant MADS-domain proteins.

Studying the evolution of MADS-box genes the question arises as to when during evolution the MIKC-type genes formed and what kind of functions they exerted before some of them became the „molecular architects of the flowers".

The isolation of MIKC-type MADS-box genes from the ferns *Ceratopteris* and *Ophioglossum* led to the conclusion that these plant specific MADS-box genes must have been originated more than 400 MYA, because at that time the lineages leading to the higher plants and the ferns separated. Phylogenetic analysis of the fern MADS-box genes showed that these genes are homologs, but not orthologs of the floral homeotic genes. Due to the fact that a transformation protocol for ferns is not yet available and because the genes are expressed quite ubiquitously, it proved difficult to get clues on the genes´ functions.

In order to get information about functional aspects of MADS-box genes in non-seed plants we decided to work with the moss *Physcomitrella patens*, because this is the only land plant which offers the possibility to knock out genes via homologous recombination to study gene functions as revealed by mutant phenotypes.

We isolated three different MADS-box gene cDNAs - K71, K445 and KH52 - from *Physcomitrella* via 3´-RACE and screening of a cDNA-library. All three cDNAs represent single loci genes in the moss genome, as shown by high stringent Southern blot hybridizations.

The conceptual *Physcomitrella* MADS-domain proteins have the typical MIKC- structure. However in phylogeny reconstructions the moss genes do not belong to any of the well defined subfamilies of MADS-box genes known from higher plants or ferns.

The cloning of the genomic loci via genomic PCR and 5´-RAGE resulted in two types of exon-intron organizations. While K445 and KH52 share the common genomic structure of MADS-box genes from the MIKC-type, K71 shows an increased number of exons encoding the I-domain of the conceptual protein.

Using K445- or K71-cDNA as probes for genomic Southern hybridization under moderate condi-

tions we could estimate a number of 5 to 8 related genes of both types of MADS-box genes in *Physcomitrella*.

For PEG-mediated protoplast transformation we constructed gene-specific knock-out vectors for K71 and K445 based on their cDNA-sequences. The first resulting transgenic mosses are currently being characterized molecularly, physiologically and morphologically.

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Expression patterns of floral homeotic gene homologs in the moss *Physcomitrella patens*, and its implication to the evolution of reproductive organs in land plants.

Rumiko Kofuji[1, 2] and Mitsuyasu Hasebe[1, 3, 4]
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Correspondence to M. Hasebe (e-mail: mhasebe@nibb.ac.jp)

Land plants form reproductive organs in both sporophyte and gametophyte generations. A flower is a sporophytic reproductive organ of angiosperms, composed of sepals, petals, stamens, and gynoecium (s). Haploid cells, mega and microspores are formed by meiosis in male and female sporangia, pollen sacks and nucelli. Both male and female gametophytes are reduced to few cells and haploid reproductive organs, such as archegonia and antheridia observed in lower land plants are not formed. Other land plants including gymnosperms, pteridophytes, and bryophytes have simpler diploid reproductive organs than angiosperms. A sporangium of gymnosperms is covered with a single integument instead of two integuments and a carpel of angiosperms. In pteridophytes and bryophytes, a sporangium is naked on a leaf or stem. On the other hand, lower land plans have more complex haploid reproductive organs, such as archegonia and antheridia. Archegonia are formed in gymnosperms, ferns, and bryophytes, and antheridia develop in the latter two groups. In Bryophytes, gametophytes with archegonia and antheridia are dominant, and sporophytic generation is epiphytic to gametophytes. In addition to the evolution of reproductive organs in each generation, evolution of morphological differences in sporophytic and gametophytic generations has not been well studied (1, 2, 3).

The development of floral organs is mainly regulated by the members of the MADS-box gene family whose members are transcription factors containing conserved MADS and K domains. MADS-box genes of angiosperms are divided into more than 10 groups based on the gene tree, and three of them have specific functions as homeotic selector genes of floral organ development, named A-, B-, and C-function genes. Studies of MADS-box genes in gymnosperms (4) and ferns (5) revealed that MADS-box genes are expressed in diploid reproductive organs in gymnosperms and ferns, although they are not specified as in angiosperms. This suggests that specification of MADS-box gene expression and function was important for the evolution of more complex diploid reproductive organs in land plant lineage (reviewed in 1, 2).

To further investigate the original function of MADS-box genes and their evolution, we characterized MADS-box genes from the moss *Physcomitrella patens*. Three MADS-box genes (*PpMADS1, 2, and* 3) were isolated from cDNA of *P. patens* by the 3'- and 5'-RACE methods using the MADS domain specific degenerate primers. Two of them lack K domain that play important roles in protein-protein interactions of MADS-box genes, suggesting that these genes are not functional. By the northern analyses, expression of *PpMADS1* was not well detected in both

protonemata and gametophores without antheridia and archegonia. Using the homologous recombination, a GUS reporter gene was connected to the 3' end of *PpMADS1* gene, and the expression was observed. In developing antheridia, GUS expression started at the spermatogenous cells just after the differentiation of antheridial jacket cells and spermatogenous cells. The expression was continued until the maturation of spermatozoids, suggesting that *PpMADS1* gene is involved in the development of spermatozoids. The *PpMADS1* expression was also observed in an egg cell in the archegonium.

According to these patterns of expression, we hypothesized that the MADS-box gene(s) was originally involved in the development of reproductive cells in haploid generation. During the course of evolution, the number of MADS-box genes presumably increased by gene duplication, and then some of them were recruited into the development of sporophyte in a vascular plant lineage, and further elaborated as a homeotic selector gene of floral organs in angiosperm lineage.

References

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3) Aso, K., Kato, M., Banks, J.A. and Hasebe, M. 1999. Characterization of homeodomain-leucine zipper genes in the fern, *Ceratopteris richardii* and the evolution of the homeodomain-leucine zipper gene family in vascular plants. Mol. Biol. Evol. 16: 544-552

4) Shindo, S., Ito, M., Ueda, K., Kato, M. and Hasebe, M. 1999. Characterization of MADS genes in the gymnosperm *Gnetum parvifolium* and its implication on the evolution of reproductive organs in seed plants. Evolution and Development 1 : 180-191.

5) Hasebe, M., Wen, C.-K., Kato, M. and Banks, J.A. 1998. Characterization of MADS homeotic genes in the fern *Ceratopteris richardii*. Proc. Natl. Acad. Sci. USA 95: 6222-6227.

Gene targeting of a *rop/rac* homologue in *Physcomitrella patens*

Nathinee Panvisavas, Celia Knight, Andrew Cuming, David Cove, (University of Leeds, UK) and Ralph Quatrano (University of Washington, St Louis, US)

We are using a low copy number sequence showing homology to the *Arabidopsis thaliana Rop* (*Rho*-related GTPases from higher plants) genes of higher plants to investigate factors affecting gene targeting in moss. *Rop1At*, a member of the *Arabidopsis thaliana rop* gene family, is proposed to play a predominant role in the regulation of polarised cell growth in pollen (Li, *et al.* Plant Phys. 1998, 118:407-417). It is possible therefore that a targeted knock-out of this gene in *Physcomitrella* may present a morphological phenotype which will assist in gene functional analysis.

A 1-kb full length cDNA with homology to *Rop1At*, was identified from the cDNA library and homologous genomic clones isolated subsequently. Replacement transformation vectors were constructed for both cDNA and gDNA sequences. The mrac transformation vector contained an *npt*II selectable marker flanked by approximately 400 and 600 bp of cDNA. The grac transformation vector contained approximately 1 kb of genomic DNA either side of the *npt*II gene. PEG-mediated transformation of protoplasts using both supercoiled and linear fragments of the grac clone have generated 21 (out of 41) targeted transgenics from the linear construct and 8 (out of 35) targeted transgenics from the supercoiled plasmids. The analysis of these transgenics will be discussed.

MOSS: A Genetic System to Study Cellular Polarity

Patricia A. Carneiro and Ralph S. Quatrano,
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The generation of cellular polarity and asymmetry represent fundamental processes that are found throughout the development of prokaryotes and eukaryotes. The mosses *Physcomitrella patens* and *Ceratodon purpureus* are ideal model systems for studying plant development because of the ability to generate mutants with altered polarity, to genetically transform cells and to replace alleles by homologous recombination.

The establishment of cell polarity can be studied by assaying the responses of the apical cell of moss protonemal filaments to environmental stimuli such as light and gravity. Previous efforts to isolate mutants impaired in these responses have been quite successful. With respect to the gravity vector, two classes of mutants have been isolated in *Ceratodon purpureus* using either UV or gamma irradation: *wrong-way-response* (wwr) mutants, and, the agravitropic mutants. The *wwr* mutants are able to align their axes with the gravity vector properly, but have an abnormal polarity, i.e. protenemal growth toward gravity instead of away from gravity. The agravitropic mutants are impaired in their ability to align their axes properly, i.e. protenemal growth is random with respect to the gravity vector.

Our goal is to identify the mutant gene(s) responsible for each phenotypic class. To date, no technique has been demonstrated to successfully tag genes at random within the moss genome. Thus, it is of extreme importance to develop such techniques and resources, which will not only aid in the identification of genes involved in the establishment of cell polarity, but will also benefit all investigators using these model organisms.

To identify the genes involved in the establishment of cell polarity, we are currently trying to develop insertional mutagenesis as a tool to inactivate genes using the mariner transposable element (Mos1) of *Drosophila* and its respective transposase. In addition, activation tagging, using a 4X 35S enhancer element which was developed for used in *Arabidopsis thaliana* (Pl. Physiol. 122: 1003-13, 2000), is now being developed in both *Physcomitrella* and *Ceratodon*. Thousands of individually transformed plants from the insertional mutagenesis and activation tagging lines will be screened for the *wwr* and/or agravitrophic phenotypes. We will report on the progress of this research.

The Role of Cell Expansion in the development of *Physcomitrella patens*

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Aims of research

We want to critically test classical ideas on the interactions of cell expansion and division in plant development, and to investigate the interdependence of cell expansion and cell division orientation and their relative roles in defining daughter cell fate.

When a (haploid) meiospore of P. patens germinates, it first develops into a branched network of filaments, the protonema. These filaments are only unicellular in diameter and extend by tip growth. However, side-branch-initials (SBIs) formed on the filaments have the potential to swell (expand) and develop into buds and subsequently into leafy shoots, the gametophores. These structures constitute the adult gametophyte and grow by isodiametric expansion of the cells (Cove, 1992).

This transition from the juvenile to the adult gametophyte serves us as a well-defined system, where we aim to experimentally influence cell expansion. Our molecular tools are small extracellular proteins called expansins, which mediate cell expansion apparently by modulating cell wall extensibility (Cosgrove, 1996; McQueen-Mason *et al.*, 1992).

Our goal is to generate transgenic mutants in which expansin is either overexpressed or knocked out. Then we aim to establish if the altered expression pattern of expansins (and thus altered cell wall extensibility) leads to changes in adopted developmental fates of the SBIs.

Identification and characterisation of *PpExp1*

An RT-PCR approach was undertaken using degenerate heterologous primers derived from rice a-expansin sequences. This led to the isolation of a 460 bp. cDNA fragment. The RACE (Rapid Amplification of cDNA Ends) technique enabled us to obtain a full-length cDNA clone of 1.2 kb. The latter contains an ORF coding for a protein of 262 amino acids and a predicted molecular weight of 28.4 kDa, which displays 65 to 70 % sequence identity and 80 % sequence similarity at the amino acid level to other known expansins.

Southern Blot data generated by hybridising genomic DNA to a labelled probe derived from the partial cDNA sequence surprisingly suggests that the expansin gene family is very small in *P. patens*. This finding contrasts with the many expansin sequences from other species accumulating in the databank (Cosgrove, 2000; Shcherban *et al.*, 1995).

Northern Blot analysis of RNA extracted from *P. patens* tissue treated with various phytohormones indicates that *PpExp1* is expressed at low level under all circumstances tested.

Knockout (KO) and overexpression mutants

Taking advantage of the unique feature of efficient homologous recombination in *P. patens* (Puchta, 1998; Schaefer and Zrÿd, 1997) expansin KO mutants were generated. A 250 bp *PpExp1* fragment was cloned upstream (5') of a CaMV 35S driven neomycin resistance cassette. Another 500 bp fragment was cloned downstream (3') of the resistance cassette. Double homologous recombination events result in an exchange of the endogenous expansin gene with the resistance cassette, allowing selection of the KO mutants. Molecular analysis of the transformants will substantiate the targeted insertion of the resistance cassette (by Southern Blot and PCR), as well as the absence of the expansin transcript (by Northern Blot).

Expansin overexpression mutants were created by cotransformation of a pUC derived vector containing a large genomic insert (approximately 3.6 kb) with another pUC derived vector containing the expansin cDNA under the control of the (constitutive) rice actin1 promoter (McElroy *et al.*, 1990; McElroy *et al.*, 1991). Insertion of multiple copies of concatemerised plasmids into the genome results in stable transformants containing the expression cassette at a precise location. Three overexpression cassettes have been stably integrated in *P. patens* transgenic mutants: (A) the endogenous expansin cDNA *PpExp1*, (B) *CsExp1*, a dicot expansin from cucumber (Shcherban *et al.*, 1995) and (C) *FpExp2*, a monocot expansin from *Festuca pratensis* (B. Reidy, unpublished results).

The phenotype of the generated KO and overexpression mutants will be reported.

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Aphototropic mutants of the moss *Ceratodon purpureus*

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The phototropic response of Ceratodon purpureus filaments is controlled by the red-farred reversible photoreceptor phytochrome. Most aphototropic mutants of this species belong to one of two different classes. Class 1 mutants are defective in the biosynthesis of the phytochrome chromophore. Phototropism and other phytochrome effects can be rescued by biliverdin or phycocyanobilin. Transient expression of heme oxygenase genes which were introduced by microinjection also resulted of a rescue of phytochrome responses. These mutants were also chosen to stain phytochrome with the chromophore phycoerythrobilin, which produces a highly fluorescent adduct. Confocal laserscanning microscopy showed that the main portion of phytochrome is cytosolic, with a high mobility. A part is also found in the nucleus. Class 2 mutants are specifically defective in the phototropic response, other phytochrome responses are regulated normally. Quite interestingly, some of these mutants grow faster than the wild type. Recently another mutant was isolated that is defective in chlorophyll biosynthesis. This mutant is probably defective in coproporphyrinogene oxydase, which catalyses the formation of protoporphyrin. As a result the mutant has lower heme and phytochrome levels and does not form chlorophyll. This was further analysed physiologically.

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Poster presentations

PEP: The Physcomitrella EST Programme

Celia Knight, Andrew Cuming, David Cove, David Westhead (University of Leeds, UK) and Ralph Quatrano (University of Washington, St Louis, US)

PEP is the *Physcomitrella* EST programme: a resource for functional genomics supported by the UK Biotechnology and Biological Sciences Research Council being developed in a collaboration between Leeds University in the UK and Washington University in the US. The programme aims to deposit *ca.* 30,000 partial cDNA sequences derived from *Physcomitrella patens* in public access databases as a resource for the world plant science community to utilise the capability of *Physcomitrella* in undertaking homologous recombination-mediated gene modification. To date, approximately 10,000 sequences have been deposited in GenBank, and are also accessible through the PEP website (under construction at the time of writing). Registration on this site permits PEP users to conduct BLAST searches of the PEP sequence database, in order to identify *Physcomitrella* homologues of genes previously identified in other organisms, and to order individual clones, cDNA and genomic libraries, and vectors designed for the construction of gene knockout or gene replacement vectors.

Currently available PEP resources include cDNA and genomic libraries constructed from protonemal tissue of *Physcomitrella patens*; an auxin-treated cDNA library is also available and others are in preparation. Additionally, plasmid vectors containing an *nptII* cassette flanked by a multiple cloning site on either one or both sides of the antibiotic resistance gene are available for the construction of allele replacement and gene disruption vectors. Finally, we are providing training and support for laboratories with no previous experience of *Physcomitrella* biology, in the form of annual workshops in *Physcomitrella* methodology, and a transformation service on a part-cost basis.

This poster will present details of the programme and provide a leaflet for researchers to take away on how to use the service.

Cytokinin metabolism in *ove* mutants of *Physcomitrella*

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3) INRA Versailles, Laboratoire de Biologie cellulaire, Route de St. Cyr, F-78026 Versailles

Mutants with a strongly increased cytokinin production have been described for the moss *Physcomitrella patens* (Ashton et. al. 1979, Wang et al. 1981). These so called *ove* mutants are characterised by a large overproduction of buds.

So far, it is unclear whether changes in cytokinin breakdown, interconversion, and/or biosynthesis are responsible for the *ove* phenotype. In order to determine the underlying mechanism, studies on cytokinin metabolism were carried out using the temperature-sensitive *ove* mutant oveST25. OveST25 exhibits the same phenotype as the wild type at 15°C, but shows an *ove* phenotype at 25°C. The *ove* mutants oveA78, oveA201 and oveB300 (Featherstone et al. 1990) were also included in the study.

***** For oveST25 (grown at 25°C), cytokinin measurements showed up to 250-fold higher concentration of isopentenyladenine in the culture medium compared to the wild type (the thiamine auxotrophic strain thiA1 was used). The cytokinin overproduction is temperature-inducible and correlates with the expression of the *ove* phenotype (Fig. 1).

***** *in vivo* metabolism studies using radiolabeled isopentenyladenosine ([9R]iP) revealed the following in the analysis of culture medium:

- OveST25, grown at 25°C, showed a 3-fold increased conversion of isopentenyladenosine to the base isopentenyladenine (iP) when compared to the wild type control (Tab. 1). The increase of isopentenyladenosine conversion is temperature-inducible (Tab. 1).
- Most of the conversion product isopentenyladenine is released into the culture medium for both wild type and oveST25.
- The other mutants oveA78, oveA201, and oveB300 showed a similar increase in the riboside-base conversion (not shown).

***** *in vivo* metabolism studies analysing the tissue extracts revealed the following:

- All *ove* mutants analysed showed degradation of cytokinin to adenine and adenosine. Consequently o*ve* strains do not seem to be degradation mutants (Fig. 2).
- In the wild type isopentenyladenosine is mainly metabolized to the nucleotide isopentenyladenosine monophosphate and to the degradation products adenosine/adenine. In *ove* mutants, however, the metabolisation of isopentenyladenosine is mainly directed towards the active base isopentenyladenine which is released into the culture medium.

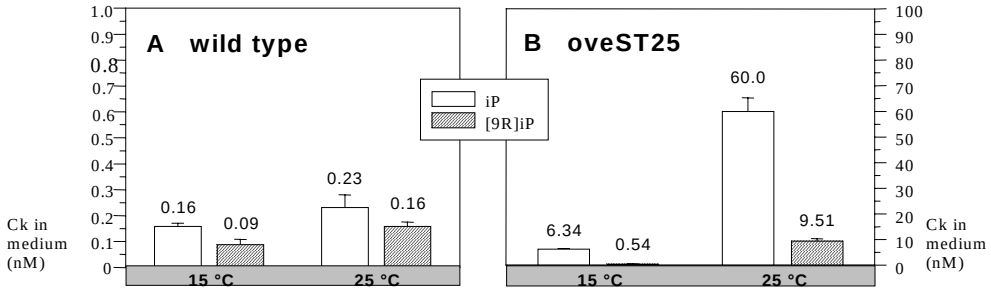
Based on these results, it can be said that the *ove* phenotype is coupled to significant changes in the cytokinin riboside metabolism. Major changes in cytokinin breakdown were not detected. Understanding the molecular basis of the metabolic deregulation in *ove* mutants could help to clarify cytokinin homeostasis in plants.

D. R. Featherstone, D. J. Cove and N. W. Ashton: Genetic analysis by somatic hybridisation of cytokinin overproducing developmental mutants of the moss, *Physcomitrella patens. Mol Gen Genet* **222** (1990), 217-224.

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

T. L. Wang, P. Beutelmann and D. J. Cove: Cytokinin biosynthesis in mutants of the moss *Physcomitrella patens*. *Plant Physiol* **68** (1981), 739-744.

Fig. 1: Thermal induction of cytokinin overproduction in the mutant oveST25. Comparison of cytokinin concentration measured by HPLC-ELISA in the culture medium of wild type (the



thiamine auxotrophic strain thiA1 was used) and its temperature-sensitive mutant oveST25. iP (isopentenyladenine) and [9R]iP (isopentenyladenosine) were measured in the medium of liquid cultures cultivated continuously at 15°C or 25°C. The age of the culture was 3 weeks; 2.1 – 3.5 mg chloronema tissue per ml medium were used. Note the different scales in A and B.

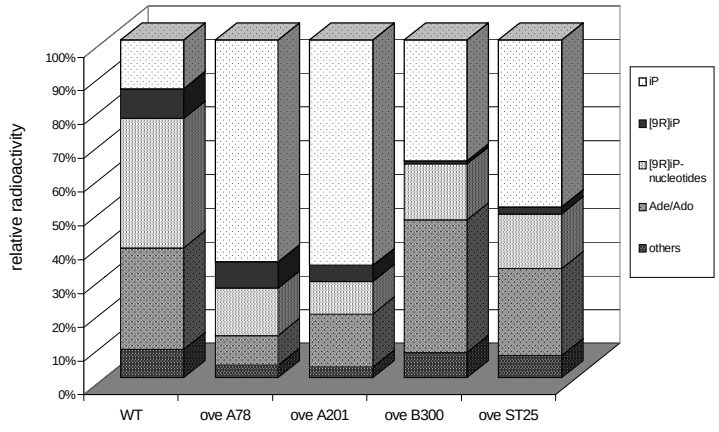


Fig. 2: Relative distribution of radiolabelled cytokinin metabolites measured in *Physcomitrella* tissue after 6 h *in vivo* labelling with tritiated isopentenyladenosine (3H-[9R]iP, 50 nM). Fresh weight: 104-160 mg per incubation.

Table 1: Relative velocities for the *in vivo* conversion of radiolabelled isopentenyladenosine to isopentenyladenine in the temperature-sensitive strain oveST25 and the wild type. Data are based on the analysis of culture medium. Tissue were grown and incubated at 15°C or 25°C.

strain	temperature	relative velocity
wild type*	15°C	1
wild type*	25°C	3.3
ove ST25	15°C	1.1
ove ST25	25°C	11

* the strain thiA1 was used as a control

Full-length cDNA activation system in *Physcomitrella patens*

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To dissect a genetic pathway screens for loss-of-function mutations by a conventional mutagen or insertional mutagenesis that leads to disrupt such a pathway, have been successfully performed in higher plants. However, it is now clear that there are limitations of the screens. They barely identify genes that act redundantly or whose loss-of-function results in embryonic or gametophytic lethality. Such genes can be still identified by applying screens for gain-of-function mutations. Recently activation tagging strategy has been utilized to this purpose in *Arabidopsis* plants. In this system, an endogenous gene is activated by enhancers or by a complete promoter from a cauliflower mosaic virus (CaMV) 35S gene, causing a dominant gain-of-function mutation. This approach has identified several new genes important for various aspects of a life cycle.

Similar approaches seem to be useful for a dissection of a genetic pathway in *Physcomitrella patens*. We've already started the screens for loss-of-function mutations in *P. patens* by developing a gene-tagging and gene-trap system, in which a *P. patens* genomic sequence was replaced with a tagged *P. patens* DNA sequence by homologous recombination, resulting in a disruption of the gene. Nishiyama *et al.* and Hiwatashi *et al.* will report details of this project in this meeting.

We will also explore a screen for gain-of-function mutations by a constitutive activation of an individual gene in the moss plant. At first, high-content and full-length cDNAs from *P. patens* are prepared based on the introduction of a biotin group into the 5' cap structure of mRNAs. The full-length cDNAs are directionally subcloned under a strong promoter, such as a modified CaMV 35S promoter. In the resultant full-length cDNA library, 5' ends of the inserted cDNAs are sequenced individually to evaluate if the cDNA represents a full-length sequence. Then we introduce each full-length cDNA clone by using a homologous recombination technique into *Pphb7* locus of the moss genome. The disruption of *Pphb7* locus revealed no morphological changes at least during gametophore development probably due to a redundancy of its gene family and the locus, therefore, is useful as a platform site. The transformed moss protoplasts are selected for antibiotic resistance and developmentally abnormal phenotype will be chosen for further analysis. We will present a detail of this approach designated as full-length cDNA activation system and discuss its potential.

Sequence of *Pphb7* will be used for a homologous recombination to introduce a cassete containing a full-length cDNA sequence and *NPTII* gene for antibiotic selection into *Pphb7* locus. P1 and P2 indicate promoters to drive the cDNA and *NPTII*, respectively.

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A partial scheme of a plasmid vector for full-length cDNA activation system

Characterization of Homeobox Genes in the Moss, *Physcomitrella patens.*

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Homeobox genes encode transcription factors characterized by the homeodomain, which binds to DNA, and play crucial roles in many aspects of development. A number of homeobox genes have been isolated from vascular plants, and are classified into several gene families based on their amino acid similarities. To understand the evolution of homeobox gene family, analyses of homeobox genes in primitive green plants are necessary. The moss, *Physcomitrella patens* was chosen because it is well developed as a model organism to characterize gene function. We performed RT-PCR with homeobox specific degenerate primers and isolated one gene (*PpKNOX1*) from the KNOX family and 10 genes (*Pphb1* to *10*) from the HD-Zip family. Angiosperm KNOX gene family is divided into two subfamilies (Class1 and 2). The Class1 genes are shown to have important roles in meristem initiation and maintenance. *PpKNOX1* has MEIKNOX domain and ELK motif common to other KNOX genes the Class1 gene and is placed at the most basal position of Class1 in the gene tree. HD-Zip gene family is divided into four subfamilies (HD-Zip I to IV). The gene tree with vascular plant HD-Zip genes and *Pphb* genes shows that nine *Pphb* genes belong to three of the four HD-Zip subfamily (HD-Zip I, II, and III) and that the gene duplication of the ancestral gene of each subfamily occurred before the split of the vascular plants and the mosses. *Pphb3* was distantly related to any other gene and was assigned a new subfamily (HD-Zip V). The HD-Zip II genes are suggested to have evolved slower than HD-Zip I genes. *Pphb2* is expressed in the protonema, *Pphb1*, *3*, *6*, *7*, *8*, *9*, *10* genes are expressed in the gametophore, and *Pphb5* gene is expressed in both as shown by Northern hybridaization. The characterization of the loss or gain of function mutants of *PpKNOX1* and *Pphb* genes are in progress and the results will be presented.

Tagged Mutagenesis and Gene-trap in the Moss, *Physcomitrella patens* by Shuttle Mutagenesis

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The moss, *Physcomitrella patens* has been used as a useful material in many fields, because of its simple body plan, easiness of gene targeting, and other reasons. Although many mutants have been reported, no method to isolate the corresponding genes was reported. We developed a gene tagging and gene-trap system in *P. patens* by using the shuttle mutagenesis technique, which has been used in the budding yeast.
The shuttle mutagenesis involves three steps: (1) Generation of *P. patens* genomic library in *Escherichia coli* (2) Insertion of a mini-transposon into the *P.patens* genomic sequence. (3) Transformation of *P. patens* with the *P. patens* genomic sequences tagged by the mini-transposon. The transformation is expected to result from a recombination between the *P. patens* DNA sequences flanking the mini-transposon and the homologous *P. patens* genomic DNA.
In 5264 tagged lines, 203 mutants with altered developmental or morphological phenotypes were obtained.
Southern hybridization showed that multiple copies of the mini-transposon were integrated in most transformants. To study how these mini-transposons were integrated, PCR analysis were performed using primers designed on the mini-transposon. Inter-mini-transposon regions were amplified in the strains into which multiple transposons were integrated, suggesting that the multiple copies were integrated tandemly at a locus.
The inter-mini-transposon regions from the mutant, TN1 were cloned and sequenced. Sixteen types of clones were obtained, of which 13 types contained *P. patens* genomic sequence derived from a single genomic clone. The genomic clone contained a putative gene, which was highly conserved among the mosses, gymnosperms, and angiosperms. Similar cloning analysis of another mutant strain, as well as the functional analysis of this gene is under progress and will be presented.

Reference:
T. Nishiyama, Y. Hiwatashi, K. Sakakibara, M. Kato, and M. Hasebe, 2000. Tagged Mutagenesis and Gene-trap in the Moss, *Physcomitrella patens* by Shuttle Mutagenesis. DNA Research 7: 9-17

Generation of gene/enhancer-trap lines in the moss, *Physcomitrella patens* and expression patterns of a reporter gene (GUS) in gene/enhancer-trap lines.

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The moss, *Physcomitrella patens* has been used as an excellent model to study molecular mechanisms of development regulated by plant hormones as the following reasons: (1) The same kinds of plant hormones as higher plants, such as auxin and cytokinin, have distinct effects on the moss development, (2) the developmental processes caused by the plant hormones can be easily observed at the cellular level, and (3) the techniques for genetics and molecular biology including gene targeting technique are well established.

In this study, in order to understand the molecular mechanism(s) of cell differentiation regulated by auxin and cytokinin, we attempted to (i) establish gene/enhancer-trap systems and (ii) identify cell-specific molecular markers.

(i) Establishment of gene/enhancer-trap systems in *P. patens*.

We introduced the promoterless or minimal promoter-fused *uidA* (GUS) gene as a reporter gene into protoplasts by the PEG-mediated transformation method and obtained stable transformants. We also obtained stable transformants by shuttle mutagenesis with a transposon containing the promoterless *uidA*. Using the gene/enhancer-trap systems, we succeeded in generating approximately 200 trap lines expressing GUS activity from approximately 6000 stable transformants. The GUS activity in the trap lines was detected in various kinds of cell-type and tissue. Thus the expression of the reporter gene can be activated in diverse cells and tissues, indicating that trap lines can be used to identify cell-type- and tissue-specific molecular markers. Many trap lines showed GUS activity in leaves or axillary hairs, indicating that the reporter gene traps some gene or enhancer active in leaves or axillary hairs at a high frequency.

(ii) Identification of trap lines that exhibit GUS activity in buds

In order to understand the molecular mechanism of the bud formation, which is mainly regulated by auxin and cytokinin, 11 trap lines that exhibit GUS activity in buds were isolated. In 4 of 11 trap lines, the GUS activity was localized to cell-type or tissue-specific manner, while the rest showed constitutive expression. The GUS activity of a gene-trap line, designated *Apicar1*, was localized to the apical cell and its surrounding cells. Thus *Apicar1* provides a useful molecular marker to analyze bud formation.

The GUS staining patterns in the trap lines we have obtained, as well as the cell-specific localization of GUS activity and the expression pattern of *uid*A mRNA in *Apicar1* will be presented.

Stable chloroplast transformation of the moss *Physcomitrella patens* using GFP as reporter.

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Plastid transformation has several advantages over nuclear transformation, but successful cases have been limited to just several laboratories and several plants. Since transformation and selection are very simple in P. patens, this moss has been used as a model plant to study developmental biology. We used this plant for plastid transformation. The vector pCT-GFP was designed to be targeted to the chloroplast genome by homologous recombination between the trnI and trnA sequences within the inverted repeat region of the plastome. Five regenerants were obtained about three weeks after one cycle of selection following particle bombardment. PCR analysis using aadA and mgfp primers identified the presence of the transgene in the chloroplast genome in all transformants indicating that one cycle of selection can be enough to sort out transgenic plastids in moss. Southern blot analysis confirmed the exact targeting to the chloroplast genome between trnI and trnA in four of the five transformants. Expression of the integrated transgene within chloroplasts was identified by immunoblot analysis using GFP antibody. The localization of the GFP protein within the chloroplasts of the transgenic plants was identified by confocal laser scanning microscopy (CLSM). The relatively low number of chloroplasts in the moss P. patens probably facilitated plastid transformation and the easy selection of transgenic plants.

Figure 1. Map of the plastid transformation vector pCT-GFP.

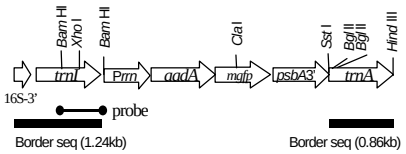
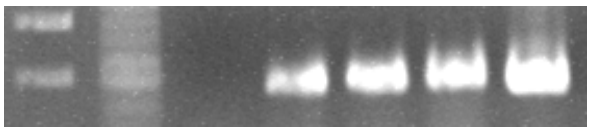
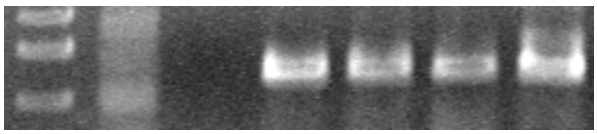


Figure 2. PCR analysis of transgenic plants. (A) PCR using aadA primers, (B) PCR using mgfp primers, (C) Southern blot analysis.

(A) 1kb 100bp WT TL1 TL2 TL3 V



(B) 1kb 100bp WT TL1 TL2 TL3 V



(C) WT TL1 TL2 TL3

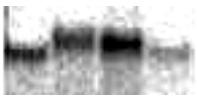


Figure 3. Immunoblot analysis of GFP expression in transgenic plants.

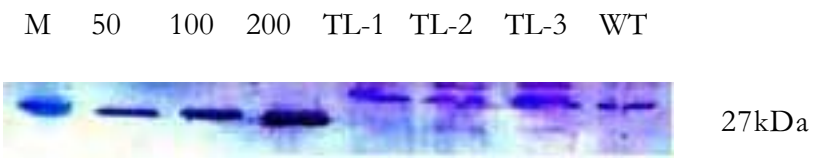
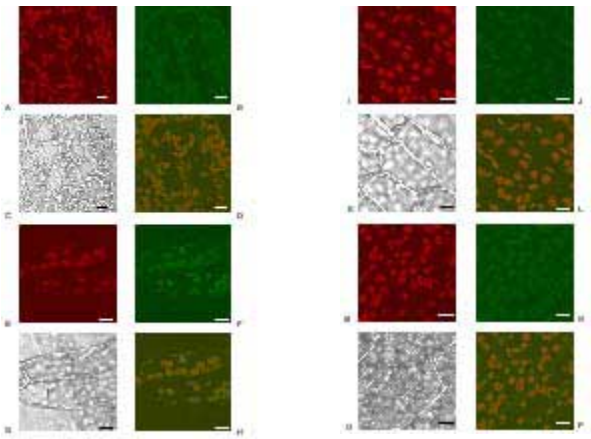


Figure 4. Visualization of GFP in transgenic plants by confocal microscopy.

Figure 5. Relative fluorescence intensity (RFI) of chloroplasts in transgenic and wild type



plants.

Table 1. The efficiency of plastid transformation in P. patens

No. of bombardments	No. of plates	No. of transformants	Efficiency (transformants/ mg DNA)	Efficiency (transformants/ plate)
Exp. 1	6	3	2	0.33	0.67
Exp. 2	7	4	2	0.29	0.50
Total	13	7	4	0.31	0.57

Effects of low frequencies magnetic field on the moss Physcomitrella patens

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Biological effects of electro-magnetic fields (EMF), is a controversial subject discussed since decades, it has been investigated up to now mainly by performing experiments on complex systems (forest, human populations) or by using medical data's. Both kind of approaches, i.e. experiments trying to put into evidence measurable effects or the use of epidemiological studies have resulted in an incredible number of publications. The epidemiological studies are dependent on too many uncontrollable factors and need too long periods of time, they often postulate that some diseases (leukemia) are related to magnetic field exposure but without any real evidence. It seems therefore urgent to concentrate tour efforts on a pure experimental biological research.

Effects due to electromagnetic fields, if they exist, are probably linked to long duration exposure; for ethical and practical reason it is impossible to experiment on humans. Experimental design should focus on the use of model plants or animals; such studies begin to show some credible results. Nevertheless the influence of the EM fields on plants has been poorly investigated. The problem is however discussed and oppositions to the installation of HV lines over forests or to the construction of radio and TV emitters near protected area have taken place.

This paper deal with the observation of the behavior of the moss *Physcomitrella patens* in a new EMF experimental setup designed to investigate the long and short time effect

1. EXPERIMENTAL SET UP

Moss plants (*Physcomitrella patens*) are grown from spores on a fully synthetic and well-defined medium in plastic Petri dishes with a diameter of about 5 cm. The test samples are placed in defined conditions of light and temperature, which permit a nearly optimal growth. The dishes are taken off at various interval of time for microscopic examination (during one hour approximately) and then placed back in their original position.

Preliminary tests have shown that electric fields have no effect on the development of the moss. Our efforts have then been concentrated on the effects of continuous and 50 Hz alternative magnetic fields and more recently on 16.6 Hz and 400 Hz fields. The fields is produced by Helmholtz coils (fig. 1), which provide reasonable field uniformity in a volume large enough for the exposing many (up to 10) plastic Petri dishes.

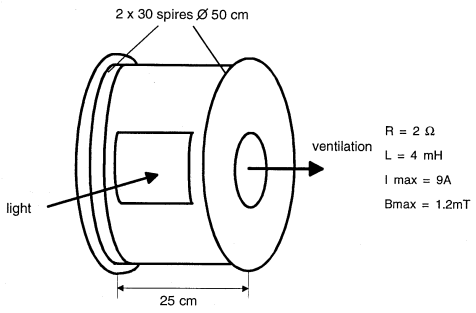
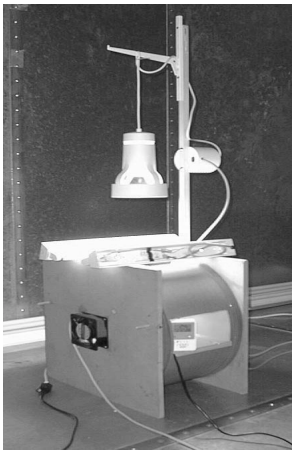


Fig. 1 – Magnetic field experimental installation. - View of a Helmholtz coil used for the production of a uniform magnetic field.



A set of 5 Helmholtz coils has been installed in a shielded cage in order to maintain the samples in controlled environment from the point of view of the magnetic field. One coil was not supplied, a second one with a DC current, and the three others with AC current in order to have, in the working volume, a magnetic field of a magnitude of 0.6, 0.9 and 1.2 mT. The samples were observed during a periods of time up to 4 weeks.

Fig. 2 – View of the Helmoltz bobbins in the experimental chambers; the light sources can be seen above it; Petri dishes can be accesed from both sides.

2. RESULTS

The following parameters have been checked to test both the effects of long time exposure (4 weeks) and the effects of the magnetic field intensity and comparison between AC and DC fields:

Number of cells at different time after spore germination (so called here - developmental stage)

The results obtained after what we would call preliminary tests have shown an influence of AC magnetic field on the growth of the moss.

The measure of the number of germinated spores after a week shows, for an exposure at a field intensity above 1.0 mT , an increase of the proportion of non-germinated spore from 2% up to 38%. Fields from 0.9 mT to 0.6 mT show a progressive decrease in this negative effect. Surprisingly, a high intensity DC field of 1.2 mT has no effect.

In order to check these first results, a more complete biometrical analysis, a longer exposure time and better experimental conditions have been used. The measurement scale has been defined as discrete development stages classified from 1 to 9. The results of 200 individual plants for each conditions are represented in the form of the average developmental stages reached at time t. Fig 3 shows a typical result obtained after an exposure of 3 weeks at different magnetic fields; the moss was cultivated in a medium containing NO3 + glucose.

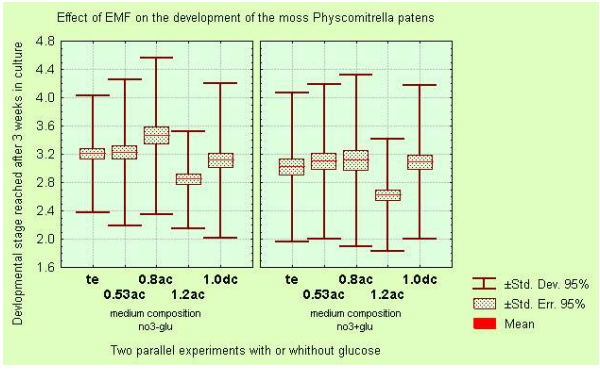


Fig. 3 – Magnetic field effects on the developmental stages reached by P.p after 3 weeks of exposure.

Those results clearly confirm the fact that an exposure to an AC field of 1.2 mT significantly disturbs the development of the moss (DC field is still not affecting development).

Some experiments indicate that a sub-optimal light intensity and quality could be responsible for those negative effects. This is tested presently in a new design of the experimental set-up in which light quality and light flux could be assessed and adapted.

3. CONCLUSION

The main conclusion of the whole study as well as other studies in progress are the facts that 50 Hz AC electromagnetic fields above 0.8 mT are affecting plant development and, surprisingly enough, that DC field up to 1.0 mT does not seems to have any effect.

In vivo observation of the actin network in the moss *Physcomitrella patens*

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According to the general model proposed by Drubin and Nelson (Drubin and Nelson, 1996), the actin network plays a central role in the establishment and maintenance of cellular polarity in eukaryotes. It reinforces the spatial cue perceived by receptors and interpreted by Rho GTPase and promotes its further propagation to microtubules and secretory pathways. Therefore, we have decided to focus our studies on actin dynamic in our attempts to develop a genetic model system to investigate plant cell polarity in *Physcomitrella*.

In plant cells, actin filaments form a net around the nucleus from which they radiate in bundles to the cell periphery where they form a branched network of cortical cables. Actin filaments also co-align with the network of microtubules with one major exception, the mitotic spindle. The actin network plays an important role in many polarity related cellular processes. In mitosis, actin filaments are associated with the microtubule preprophase band; during cell division they are associated with the growing cell plate except at the location where cell plate vesicles fuse to the mother plasma membrane. Involvement of actin filaments in tip growth is also well documented in root hairs, pollen tubes and moss filaments: in this case actin filaments are found as an apical collar like structure which is involved in targeted transport of vesicles carrying membrane and cell wall material (for references see (de Ruijter and Emons, 1999)).

The actin network in moss protonema has been studied by rhodamin phalloidin staining (Doonan and Duckett, 1988). Cortical F-actin bundles and accumulation of actin at the growing tip of filamentous cells was detected. The involvement of F-actin in tropic responses of caulonema cells of *Ceratodon purpureus* was also shown using anti-filaments drugs such as cytochalasin-D (Meske and Hartmann, 1995; Meske *et al*., 1996). Yet a complete description of the actin network in moss cells is still missing and this is probably due to the difficulties to maintain intact actin structures during cell fixation before staining. The recent development of a GFP fused to the F-actin binding domain of the mouse talin protein provides a reliable marker for *in vivo* studies of the actin microfilaments (Kost *et al*., 1998). We have transformed Physcomitrella with a 35S-GFP-talin cassette and report here a preliminary *in vivo* characterisation of the moss actin network.

In protonemal cells observed in confocal microscopy, GFP brightly labels a cortical branched network of actin bundles with an accuracy that was not observed by rhodamin phalloidin staining. F-actin bundles are aligned parallel to the axis of the cells and preliminary observation suggests that this network may be more dense in chloronema than in caulonema. Accumulation of GFP was also detected on both sides of the cell wall separating 2 adjacent filamentous cells as well as at the tip of actively growing apical cells, confirming thus previous observation suggesting that actin is actively involved in tip growth. A weaker fluorescence was also detected that surrounds the nuclei of each cell, indicating that F-actin forms a net around the nucleus. Finally brightly labelled cortical star-like structures connected with actin cables were observed in many cells. These structures correspond to microfilaments organising centres which were described in *Funaria hygrometrica* (Quader and Schnepf, 1989). Quader and Schnepf have hypothesized that those actin arrays indicate the position of the next lateral branch. So far, we

have not been able to confirm this hypothesis and we consider that they may also correspond to the site where actin polymerisation and cable formation occur. Further experiments are required to determine the function of these structures. The same features were observed in freshly isolated protoplasts and in leaf cells, but not in buds. Unfortunately, they have dissapeared in early phase of regeneration of protoplasts. In buds, GFP labelling is very weak and seems to colocalise with chloroplasts. The absence of GFP labelled F-actin in young buds may indicate that either a moss talin-like protein is highly expressed at this developmental stage, thus preventing GFP-talin to bind to F-actin, or that actin bundles are almost absent from buds: this needs to be further investigated.

The use of GFP-talin provides an outstanding tool to visualise F-actin *in vivo* in moss cells with an accuracy that is not possible to reach with standard fixation and coloration techniques. The data presented here are extremely preliminary and deserve further studies. Time lapse video microscopy will be performed to describe the dynamic of F-actin structures during cell growth and cell division. Experiments with specific inhibitors of microfilaments and microtubules will also be conducted to analyse the effect of these drugs on the observed structures. Finally we intend to use these strains to perform gene disruption experiments of the putative polarity genes isolated in the other approaches to be able to directly monitor the effect of loss of function on the F-actin cytoskeleton.

Acknowledgement

This work is supported by the Swiss National Science Foundation grant # 31-51853.97

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Using fission yeast to identify plant cell polarity genes

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Cell polarity organization during cell cycle is capital for growth regulation in living organisms. Different works on budding yeast (for review see Pruyne and Bretscher a and b) and fission yeast (for review see Gould and Simanis 1997) have permitted to define specific actors implicated in the coordination and the regulation of cytoskeleton structural elements like actin during this process. In plants, in spite of the description of mutants specifically impaired in cell polarity during mitosis (e.g. Trass et al. 1995), the genetic knowledge remains very poor.

The aim of this work is to identify genes implicated in cell polarity organization in *Physcomitrella patens* using molecular knowledge and functional opportunities that offers to us *Schizosaccharomyces pombe*, the fission yeast.

The approach used to isolate genes implicated in cell polarity processes is based on a functional screen of fission yeast overexpressing moss cDNA (Xia et al. 1996) (Figure 1). After transformation of yeast strains with a moss cDNA library, phenotypic screening is possible based on characters as differential phloxin accumulation, a marker of cell viability, morphological changes during division as well as specific staining of cellular elements (DAPI for nucleus, calcofluor for parietal compounds).

In a first time, we have transformed fission yeast strain leu 1-32 with a moss conditional expression cDNA library. From 100 000 transformed colonies, screening on characters described above permitted to identify 4 clones with a phenotype in relation with alteration of cell polarity. The corresponding moss cDNA responsible for these alterations were isolated and characterized (homologues to a GST (partial), to a cDNAs of unknown function (partial), to a rab-GAP (full length) and to a gene induced upon nodulation in *Medicago* (full length).

To obtain more specifically genes altering cell polarity, we have transformed two thermo-sensible mutants affected in cell polarity, cdc4-8 and myo2. 30000 transformants of cdc4-8 and 10000 of myo2 have been screened at permissive temperature (25°C). 4 cDNA inducing polarity defaults were isolated and characterized. All partial, the first presents high homology with *A. thaliana* protein ZW10 (unknown function), the second shows us amino acid patterns found in different animal collagen elements and the two last display short specific amino acid pattern but no specific homology with known proteins.

In the near, the possibility of homologous recombinaison in *P. patens* will permit to knock-out and overexpress these genes and assign them a definitive function.

Acknowledgement

This work is supported by the Swiss National Science Foundation grant # 31-51853.97

Litterature:

Gould, K.L. and Simanis, V. (1997) The control of septum formation in fission yeast. Gene and

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Pruyne, D and Bretscher (2000a) A. Polarity of cell growth I. Journal of cell Science 113: 365-375

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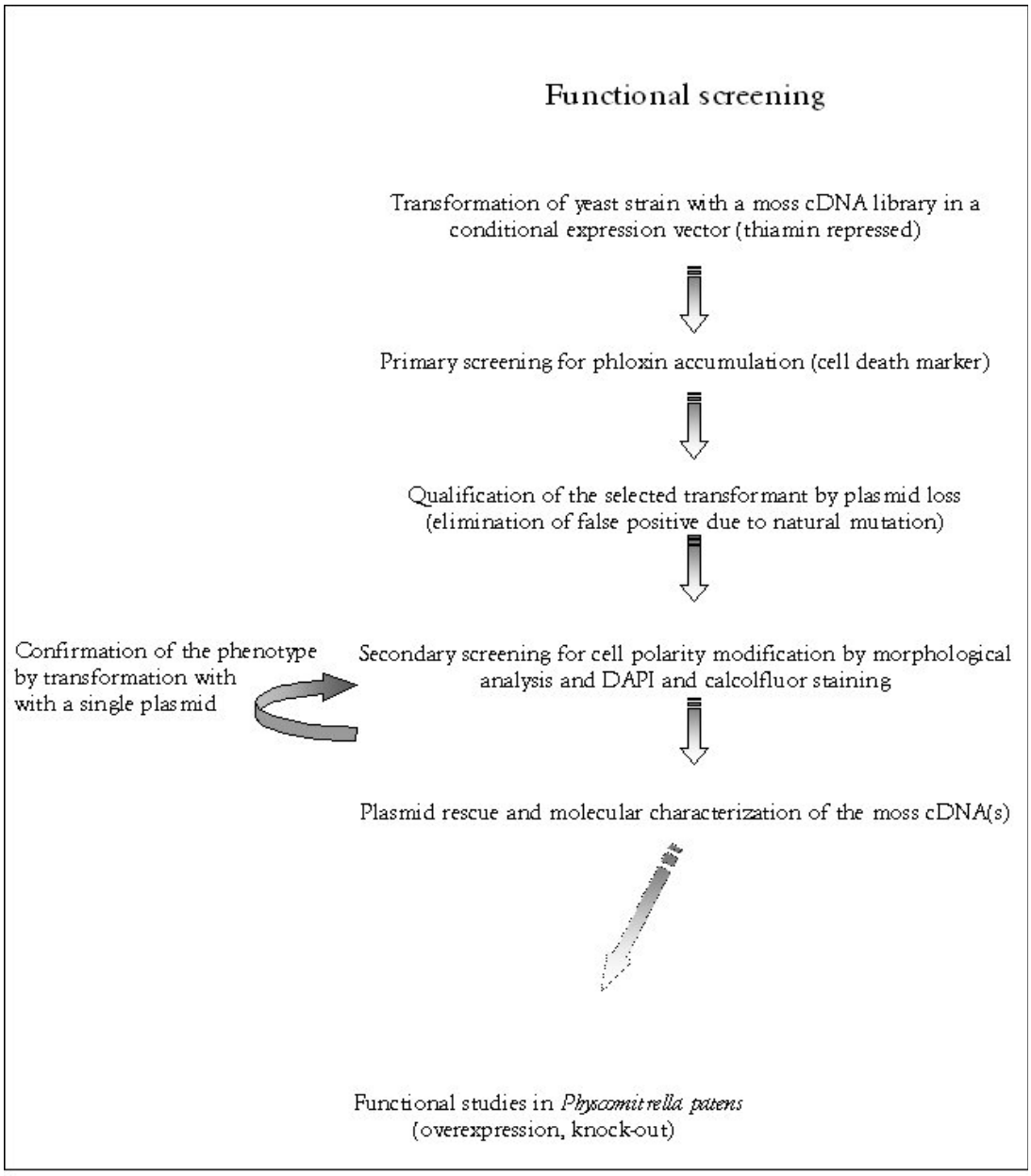
Xia, G., Ramachadran, S., Hong, Y., Simanis, V. and Chua N.-H. (1996) Identification of plant cytoskeletal, cell cycle-related proteins using Schyzossacharomyces pombe. The Plant Journal, 10: 761-769

Figure 1: General pathway to isolate genes implicated in cell polarity in Physcomitrella patens using the fission yeast Schizosaccharomyces pombe



New tools for genetic engineering of the moss *Physcomitrella patens*

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Cre-recombinase mediated excision of selectable markers and glucocorticoid inducible promoter system have been adapted to the new emerging plant model the moss *Physcomitrella patens*.

Cre-recombinase mediated excision of selectable markers

Transformation vectors used to produce knock-out mutants in Physcomitrella patens ordinarily carry a positive selectable marker M+ driven by a strong promoter. The following problems are associated with M+ usage a) the limited number of M+ available for P.patens; b) chimeric sense or antisens transcripts of the knocked-out gene due to transcriptional read-through from the M+ with unpredictable effects c) interaction with other genes by M+ promoter d) integrated M+ provides an inopportune target for subsequent transformation of the transformed strain.

In this study we tried to use the Cre-recombinase, which excises DNA regions flanked by direct repeats of its recognition lox sites (Ow 1996), to remove integrated M+. A transformation vector carrying a 35S-neo cassette flanked by 2 lox sites was used to transform *Physcomitrella patens*. Several strains were obtained in which different number of vector copies integrated in the genome. Cre-recombinase was transiently expressed in protoplasts of three of these strains (S1, S10 and S20 carrying 1, 10 and 20-30 integrated copies, respectively) and a population of up to 300 single protoplast derived colonies was screened for loss of neomycin resistance. Cre-mediated excision of the 35S-neo cassette was then confirmed by PCR and Southern blot analyses of neomycin sensitive clones. In strain **S1** the Cre-mediated excision of the M+ occurred according to the predicted model in 10 clones out of 500 (derived from 2 treatments). No neomycin sensitive clones were obtained upon the treatment of **S10** or **S20** strains so far (approx. 200 clones tested for each). Cre-treatment efficiency must be indeed diminished when multiple integrated copies of M+ have to be excised. We conclude that Cre-lox system can be used in P.patens to excise selectable markers

(or perhaps other regions of interest). The system was succesfully used so far for a single copy excisable region of 1.9 kb and the desirable clones could be easily obtained in a population of less than 1000 clones.

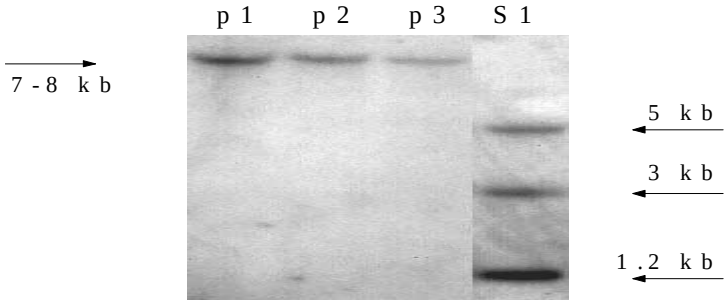

Fig. 1
Southern blot analysis of neomycin sensitive clones p1, p2, p3 and of the initial strain S1. DNA was digested with NcoI and hybridised with pBluescript + 35SNeo. In clones p1, p2, p3 the 35SNeo excision leads to the loss of 2 NcoI sites as confirmed by the presence of one band instead of three bands of 1.2, 3 and 5kb.

Glucocorticoid inducible promoter system

Few systems allowing fine gene regulation by artificial inducible promoters are available in plants. The glucocorticoid-mediated transcriptional induction system (Aoyama and Chua 1997) was adapted

to *P.patens*. It relies upon an artificial transcription factor (GVG) combining rat glucocorticoid receptor domain, herpes virus VP16 transactivation domain and yeast GAL4 DNA binding domain. In the presence of glucocorticoids (dexamethasone DEX is used in this study), it interacts with an artificial promoter based on yeast GAL4 upstream activating sequences (UAS) leading to increased transcription rates.

The moss P.patens protoplasts were co-transformed with GUS expression vector driven by UAS (UASGUS) together with GVG expression vectors either driven by a rice actin promoter pAct1 (ActGVG) or CaMV 35S promoter (35SGVG). Protoplasts were incubated for 48h in presence or absence of DEX in concentrations varying from 0 to 300 mM and GUS activity was measured. No activity was detected in protoplasts transformed without DNA (negative control). An activity ranging from 10 to 40 nmole MUG/mg prot/min was routinely observed in assays with pAct1 driven GUS expression vector used as positive control.

Best results were observed in presence of 30 – 100 mM DEX without important difference within this range. Maximum GUS activities in UASGUS – GVG system were comparable to those of pAct1 GUS and the induction factor was in range of 25-40. Some weak basal activity was also observed. Induced activities were higher in assays with pAct1 driven GVG known to be stronger than 35S promoter in P.patens.

We conclude that the development of an inducible system for P.patens shows good perspective. Maximal induced activity approaches that of pAct1 and the induction factor is about 25 - 40 which is however lower than reported for similar systems in N.tabacum and A.thaliana (see ref., up to 100 fold). All these parameters including the basal activity are yet to be assessed in constitutive mutants rather than by transient expression assays.

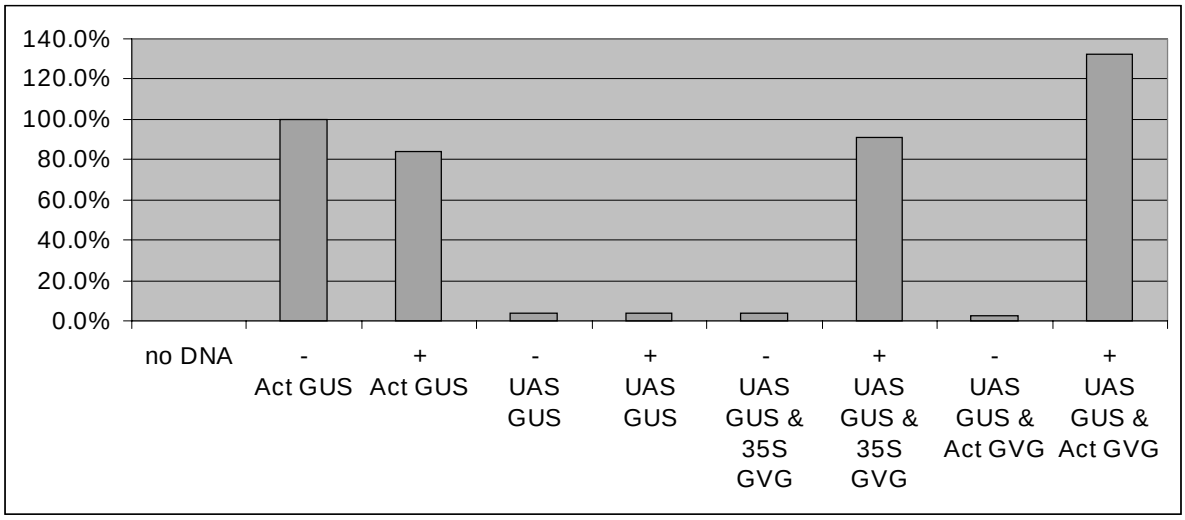

Fig 2.

Relative GUS activity measured in a typical assay. ActGUS = 100% (details see in text), minuses correspond to absence of DEX, pluses correspond to the presence of 50 mM DEX.

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Dynamics of the cytoskeleton during the polarotropic orientation of the first division of *Physcomitrella patens* protoplasts

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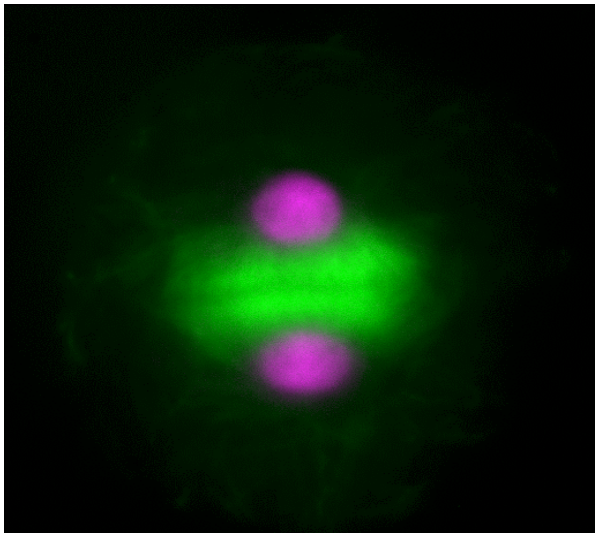
Culture of moss protoplasts is a very efficient model system for developmental studies of cell polarisation controlled by environmental stimuli. Moss cells demonstrate distinct tropic growth responses under the vectorial influence of unidirectional light and plane-polarised light. Structural reorganisation of microfilaments and microtubules mediated by phytochrome is supposed to be involved in the transduction of light signals into tropic reactions of apical cell of moss protonema. Moss protoplasts isolated from protonema appear like apolar spherical cells regenerating to give rise to development of a new moss organism beginning with the first division which potentially can be oriented in all directions. Our study was focused on the reorganisation of microtubules of the moss *Physcomitrella patens* (Hedw.) B.S.G. during the first division of protoplast under directional influence of plane-polarised light. Protoplasts were regenerated in a thin film of "top agar" layer on a cello-phane support in a glass petri dishes covered by polarising or neutral non-polarising filters, installed horizontally and illuminated from above by white light from Sylvania fluorescent tubes with resultant photon flux 16 mmol $m^{-2}s^{-1}$ in 16:8 h photoperiod. Fixation and immunolabeling of protoplasts was conducted inside the agar layer in accordance with our original adaptation of protocols. The 5x10 mm pieces of agar films with protoplasts mounted between cover-slip and glass slide were analyzed with microscope equipped with cooled Photometrics AT200 CCD Camera System.

Fig 1 Dividing moss protoplast (in green microtubules-tubulin, in pink nuclei)



In freshly isolated protoplasts microtubules are distributed in a cytoplasm as network of long bundles. They enmesh nucleus and chloroplasts, occupy a cortical layer and extend from the nuclear

surface to the cell periphery. Microtubules do not display oriented rearrangement with respect to electrical vector of polarised light before the onset of cell division. Protoplasts retain a spherical form until cytokinesis. During metaphase, chromosomes congress at the middle of spindle. The metaphase plate is equidistant from the poles and oriented perpendicular to spindle axis. The spindle is characterised by broad poles where microtubules form sub-groups focused at the "mini-poles". The anaphase spindle possesses narrow poles connected with polar and kinetochore microtubules. During mid anaphase kinetochore microtubules shorten and in the equatorial zone of spindle the concentration of microtubules increases. The arrangement of microtubules of opposite polarity in the mid-zone of spindle can be considered as the onset of phragmoplast formation. At the late anaphase, spindle sustains symmetrical form and possesses wide poles with equator perpendicular to spindle axis. Microtubules in the mid-zone extend up to groups of chromosomes segregated near spindle poles. During mitosis spindle maintains symmetrical form and mainly occupies the central region of protoplast which remains spherical and does not display any polarity features except mitotic apparatus structure. At a telophase new membranes form around the daughter nuclei . A newly-formed phragmoplast is positioned between nuclei. The phragmoplast microtubules are anchored on the proximal surfaces of daughter nuclei, from other sides sparse microtubules run out in all directions and reach the cell periphery. The newly forming cell plate is located between anti-parallel microtubules at the equatorial zone. The phragmoplast grows by centrifugal expension up to the cell periphery. The growing mid phragmoplast is discoid (Fig. 1, bar – 10 mm) and differs from spindle-shaped early one. In some cells the late phragmoplast partitions the cytoplasm of nearly spheroidal mother cell into two equal parts, in others it unequally divides the cell. After the cytokinesis the mirotubules in daughter cells are distributed throughout the cytoplasm, they enmesh the nuclei and chloroplasts and form a dense network at the cortical layer. The two-cell complex becomes pear-shaped. The narrow cell extend and divide and produce chloronema filament with cross-walls perpendicular to its axis. The character of microtubules reorganisation during the first division of moss protoplast in polarised light and in non-polarised light is similar except the orientation of spindle and phragmoplast. The metaphase plate and equatorial plane of anaphase spindle are oriented at right angle to the electrical vector of polarised light. In protoplasts incubated in non-polarised light spindle is oriented in all directions. The equatorial plane of phragmoplast inherits the orientation of metaphase plate and is also aligned perpendicular to the electrical vector. In protoplasts regenerated in non-polarised light the phragmoplast is aligned at random angles. In the darkness protoplasts do not divide. Nuclei and chloroplasts are localized near the cell periphery and surrounded by microtubules. Our observations provide evidence for the involvement of dichroic pigments in the process of transduction light signal to moss cell system responsible to orientation of division plane. One of serious candidates to this role is phytochrome which apparently could be firmly anchored in the cell cortex with predominant orientation of one chromophore to other. As a result a gradients of photon absorption will be established in protoplasts during incubation in plane polarised light with extreme meanings of absorption at the polar regions of protoplast or in its equator. Thus, polar or equatorial excitation of chromophores might stimulate photochemical reactions finally resulting in physiological polarisation of the cell and imprinting of a cell division plane. Possible phosphorylation of cortical substrates by plant analogue of $p34^{cdc2}$ at the "zone of attraction" of expanding phragmoplast marked by preporophase band of microtubules (Sundarsean and Colasanti, 1998) allows us to suppose that orientation of cell division plane in moss protoplast is mediated by phytochrome in cooperation with cyclin-dependent kinases. To test this hypothesis further immunofluorescence observations are necessary and our unicellular model can be used in these experiments.

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Evolution of plastid division

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In contrast to the plant cell itself plastids divide like bacteria by constriction. At the site of constriction the so-called plastid division rings are formed as structures that can be detected by electron microscopy. The molecular composition of the plastid division rings is not yet identified. However, in bacteria some of the components involved in cell division are quite well characterised: the crucial step at the beginning of constriction displays the polymerisation of FtsZ molecules to a filamentous ring structure, the Z-ring. FtsZ shares striking features with eukaryotic tubulins, among them binding of GTP and GTPase activity as well as a common three dimensional structure and in vitro polymerisation to filaments. Homologues of bacterial FtsZ proteins have been identified in some plant species. The targeted disruption of *Physcomitrella patens ftsZ* as well as antisense suppression in *Arabidopsis* resulted in the inhibition of chloroplast division within the transgenic plants (1, 2). These results indicated the involvement of FtsZ in plastid division, but the exact function of plant *ftsZ* genes remains to be shown. Whereas most bacteria contain only one *ftsZ* gene, in some archaea and plants two different *ftsZ* cDNAs could be isolated. *In vitro* import studies with isolated chloroplasts suggested that *Arabidopsis* FtsZ1 protein is imported into plastids while AtFtsZ2 seemed to stay in the cytoplasm (2). Hence it was concluded that FtsZ1 could form an inner and FtsZ2 an outer plastid division ring.

Here we report the isolation and characterisation of two different nuclear-encoded *Physcomitrella ftsZ* genes. A phylogenetic analysis revealed that both gene products cluster in the same clade with *Arabidopsis* FtsZ2. For a more detailed characterisation of *Physcomitrella* FtsZ function we analysed subcellular localisation and influence on plastid division of recombinant In contrast to previous results with *Arabidopsis* FtsZ2, both *Physcomitrella* FtsZ proteins were imported into chloroplasts. Overexpression of recombinant *ftsZ* showed dose-dependent effects on plastid division This

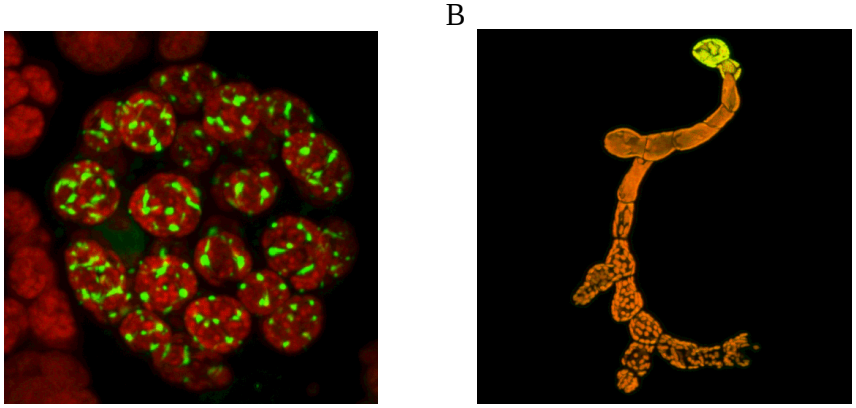

Abb. 1: Import into chloroplasts and inhibition of plastid division by overexpression of *Physcomitrella* FtsZ proteins. (A) Protoplast two days after transfection with FtsZ1-GFP. Object size: 35x35x28 µm, magnification: 1000x. (B) Protonema ten days after transfection with FtsZ2-GFP. Object size: 351x351x16.5 µm, magnification: 200x

correlation was also observed before in *E. coli* On the one hand, weak overexpression (indicated by low GFP fluorescence) resulted in a reduced chloroplast size. On the other hand, strong overexpressionan caused a transient inhibition of plastid division resulting in one giant plastid per cell. This phenotype was quite similar to the previously shown FtsZ-knockout phenotype (1). Within the growing protonema filament the inhibition of plastid division disappeared in parallel to decrease of GFP fluorescence. The dose-dependent interference with plastid division demonstrates an important role for each of the *Physcomitrella* FtsZ proteins within the division process.

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Use of *Physcomitrella patens* to study the function of the plant specific WRKY transcription factor family

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WRKY proteins comprise a new class of transcription factors that are confined to the plant kingdom. The WRKY domain, a 60 amino acid region, gives its name to all members of this superfamily of transcription factors. The WRKY domain can be divided into the N-terminal part containing the conserved amino acid sequence WRKYGQK and a zinc-finger-like motif at its C-terminus. cDNAs encoding WRKY proteins have been cloned from sweet potato (SPF1), wild oat (ABF1, 2), parsley (*Pc*WRKY1, 2, 3), tobacco (*Na*WRKY1, 2, 3) and Arabidopsis (ZAP1) due to their ability to bind specifically to the DNA consensus sequence (T)TGAC(C). This target motive of WRKY proteins, the W Box, is a conserved promotor element. W boxes were identified as functionally important elements in the regulatory regions of several defense response genes thereby implicating WRKY transcription factors in the pathogen-triggered signal transduction cascade leading to active plant defense. Moreover, altered expression levels of WRKY genes themselves can be correlated with pathogen attack, wounding and other stress responses.

WRKY proteins can be structurally divided into three groups based on the number and type of their WRKY domains: Proteins with two WRKY domains belong to group I and those with one WRKY domain belong either to group II or III. Group II and III WRKY proteins can be distinguished from each other mainly by differences within their zinc-finger motif.

The two WRKY domains in group I members appear to be functionally distinct as sequence specific binding to the target DNA element could only be obtained with the more C-terminal one. This suggests that the N-terminal domain might either participate in the binding process by altering specificity or it may mediate protein-protein interaction, a known function of zinc-finger-like domains.

A subfamily of group II WRKY proteins contains a second highly conserved amino acid motif outside the WRKY domain, the so called HARF domain. Although no defined function has be assigned to this domain yet, the absolute conservation of this motif (GHARFRR) within this subfamily suggests its involvement in determining some form of specificity. Outside the conserved regions the overall sequence homology of the WRKY proteins is low, possibly contributing to the specificity of signaling in various pathways.

While the overwhelming mass of sequence information available gives insight into the molecular basis of gene products, the biological relevance or physiological role of WRKY proteins in their context still needs elucidation. One suitable approach to learn about possible protein function in plants is the generation and characterization of loss-of-function mutants. Despite the fact that database searches revealed an abundant number of WRKY transcription factors, with at least 80 members in the *Arabidopsis* genome, surprisingly few mutants have been isolated by reverse genetics approaches.

Although WRKY genes are rather distinct, the similarity of the WRKY domain of higher plants is high enough that degenerate primers can be made using the sequence information available, in order to isolate WRKY genes from any plant species. We are interested in the function of WRKY proteins and in addition, whether they occur in all plants. Therefore we used this PCR based approach to isolate WRKY genes from *Physcomitrella patens* opening up the possibility of targeted disruption of the gene sequence by homologous recombination. In total, nine different WRKY gene fragments have been obtained by this screen demonstrating that WRKY genes belong to an essential subset of plant genes dating back at least 350 million years in time.

The isolated WRKY genes from *Physcomitrella patens* offer an excellent opportunity to study the role of different WRKY transcription factors in diverse processes conserved between lower and higher plants. It is noteworthy that many intron / exon boundaries seem to be conserved. In addition, the highly conserved amino acid sequences within the WRKY and HARF domains of all Group II / HARF proteins, even across plant orders, marks the relevance of this subfamily of transcription factors: Sequence comparison of the WRKY/HARF domains between the *Physcomitrella patens* and *Arabidopsis thaliana* counterparts revealed that they are more closely related to each other than this *Arabidopsis* subfamily to the other *Arabidopsis* WRKY genes. For this reason our interest for the first WRKY "knock-out" mutants is focused on this subfamily of WRKY genes.

PCR based screening of a *P.patens* protonema cDNA library resulted in the identification of a clone, PpWRKY20, which yielded enough sequence information to design a construct for protoplast transformation. A stable "knock-out" mutant was regenerated and compared with wildtype moss. Presently, experiments are on the way to establish whether these plants have a detectable phenotype or not. As we know that there is a response of WRKY gene expression to various biotic and abiotic stresses we plan to establish a testing scheme designed specifically to the capabilities of *Physcomitrella patens*.

Recent sequence data shows that a second *P.patens* WRKY gene (*Pp*WRKY5) is nearly identical within the obtained sequence to *Pp*WRKY20 with an identical WRKY domain sequence at the amino acid level. Hence, functional redundancy may exist meaning that more than one related WRKY gene will have to be disrupted before gene function can be determined.

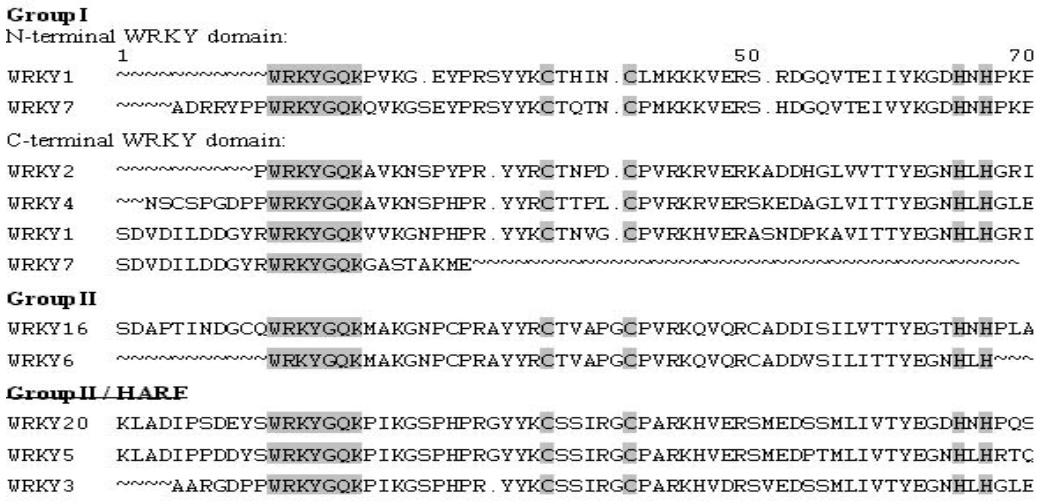

Fig.1: Alignment of the deduced WRKY domains from isolated fragments of *Physcomitrella patens* WRKY genes. The conserved WRKYGQK and the zinc finger motif are highlighted in grey.

Development of the moss *Ceratodon purpureus* as a model for plant biotechnology

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We are interested in developing a model system that can be used to understand problems important in agriculture. Mosses such as *Physcomitrella* possess a number of characteristics which make it an attractive model including: ability to grow on defined agar media on petri dishes; predominant haploid state; easily transformable with foreign DNA; and high efficiency homologous recombination. The ability to knock out genes either by targeted disruption or gene replacement provides the opportunity to assess function of specific genes of interest identified based on sequence analysis. We are interested in developing tools such as transposon systems and testing for homologous recombination in the moss *Ceratodon purpureus*. An outline of current and future experiments will be presented.

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Synthesis and Function of Membrane Lipids in *Physcomitrella patens*

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The current project which was initiated in July 1999 at the Institute of Plant Physiology in Berlin aims at the elucidation of lipid metabolic pathways in plants using Physcomitrella patens as a model system. The pathways of lipid biosynthesis are known to form a complex network which leads to numerous lipids of different classes such as neutral lipids, glycolipids and phospholipids (reviewed by Ohirogge and Browse, 1995). The goal of our project is to asses the importance of single reactions in this metabolic network. Are there still unknown pathways which can bypass a block in one of the major lipid biosynthetic pathways? To which extent does a given pathway contribute to the total synthesis of a specific lipid? These questions can be addressed using the unique knock-out system of Physcomitrella patens.

Mosses are known to possess most of the lipids and fatty acids present in higher plants (Kunzler and Eichenberger, 1997). In addition, some moss species contain certain non-phosphorous polar lipids and fatty acids absent from higher plants. The suitability of Physcomitrella for the isolation and characterization of genes involved in lipid metabolism has been demonstrated (Girke et al., 1998). By blocking selected reactions by targeted gene-disruption, followed by biochemical and physiological analysis of the stable transformands, we hope to obtain a deeper insight into the pattern of biosynthetic pathways and their regulation in plant lipid metabolism.

Step 1: Screening for genes involved in lipid metabolism
Different cDNA-libraries of Physcomitrella patens were screened with several expressed sequence tags (ESTs) representing genes of lipid metabolism of Arabidopsis thaliana. In addition, ESTs from Physcomitrella with sequence similarities to known lipid genes were directly obtained from the public Physcomitrella EST Program (see below). For incomplete clones, the missing 5' end will be isolated by re-screening the Physcomitrella library or by RACE-PCR. At present, our project is focussing on two clones with sequence similarities to Phosphatidylserine Decarboxylase (PSD) and Diacylglycerol Kinase (DAGK).
Step 2: Generation of knock-out-constructs
Knock-out constructs will be generated by the introduction of a kanamycin-resistance (nptll) cassette into the digested cDNA. Only the linear construct containing the kanamycin cassette and flanking sequences of the cDNA will be used for Physcomitrella transformation (Girke et al. 1998).
Step 3: Transformation of Physcomitrella protoplasts
Physcomitrella protoplasts are currently being transformed with the knock-out constructs. Regeneration and selection on kanamycin is underway.
Step 4: Confirmation of gene function
Because the Physcomitrella cDNAs were solely selected based on their sequence similarity to known lipid genes, we will have to confirm the enzymatic function of the corresponding protein by heterologous expression in Escherichia coli.
Step5: Biochemical analysis of Physcomitrella knock-out lines
Transformed lines carrying a knock-out mutation in one of the target genes will be used for biochemical analysis of lipids by gas chromatography and thin-layer chromatography. Furthermore, feeding of radioactive precursors will be employed to compare the fate of substrates in wild-type and mutant lines.

References and Acknowledgments

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