High throughput metabolic screen of *Physcomitrella* transformants

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Abstract. A large-scale metabolic screen was performed for 51,180 targeted knockout mutants of the haploid moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. The growth ability of each mutant was compared to the wild type. Plants were cultured on a minimal medium which contained only macroelements, as well as on a supplemented medium, additionally containing microelements, glucose, vitamins, ammonium tartrate, adenine, peptone and Na-palmitic acid. The screen resulted in the identification of 20 (0.04 %) auxotrophs. Medium supplementation tests were performed for five auxotrophs, which showed no growth on minimal medium, but were rescued on supplemented medium. One vitamin-deficient mutant was identified as p-aminobenzoic acid auxotroph, three plants were nitrate assimilation deficient mutants and one transformant showed undefined growth requirements. The screen resulted further in the identification of two physiological mutants, exhibiting an albino phenotype on minimal medium but a green wild type phenotype on the supplemented medium. The culture of both albinos under low light intensities could not prevent bleaching, revealing that the missing production of chlorophyll was not caused by light sensitivity. Astonishingly, the supplementation of the minimal medium with selected compounds of the supplemented medium did also not prevent bleaching. Moreover, both mutants produced green protonemata, even if single substances of the supplemented medium were lacking. It was concluded that a complex network of interactions related to photosynthesis in *Physcomitrella* was disturbed. This is the first detailed study of auxotrophic and albino *Physcomitrella* mutants produced by transformation-mediated gene disruption. The described physiological mutants provide valuable resources for the identification of essential gene functions of plant metabolism.

Keywords. Albino, auxotrophic mutant, metabolic mutant, bryophyte, moss, systems biology, *Physcomitrella patens*.

The moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. is able to integrate foreign DNA by homologous recombination at a rate which is several orders of magnitude higher compared to seed plants, allowing a routine production of targeted gene knockouts (Girke et al. 1998; Girod et al. 1999; Reski 1998, 1999; Schaefer 2001; Schaefer & Zrÿd 1997; Strepp et al. 1998). Collections of such knockout

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mutants provide various different phenotypes as basic material for forward genetics approaches for gene isolation and their molecular characterization (Egener et al. 2002; Nishiyama et al. 2000; Schween et al. 2005a).

For *Arabidopsis* it was already shown, that the knockout approach to plant metabolism is enormously powerful (Thorneycroft et al. 2001). Knockout mutants with metabolic defiencies, e.g., albinos, were successfully used to identify and characterize gene functions (Carol et al. 1999; Motohashi et al. 2001). However, there are hints that some kinds of auxotrophs may not be recovered in the rather complex seed plant *Arabidopsis* (Patton et al. 1998). A huge contribution to gene identification is therefore expected from knockout mutants of the lower land plant *Physcomitrella*. The majority of genes is significantly conserved between moss and seed plants (Reski 2003). Moreover, the average gene family size in *Physcomitrella* is smaller than in *Arabidopsis* (Rensing et al. 2002), enabling the precise establishment of gene/function relationships (Holtorf et al. 2002). In addition, thousands of genes not known from current databases have been found for *Physcomitrella* (Rensing et al. 2002, 2005), which is therefore assumed to provide a rich source of novel genes. This is especially true for genes involved in different metabolic pathways, as recent annotation of *Physcomitrella* expressed sequence tags identified an exceptional high proportion of these genes in the moss transcriptome (Lang et al. 2005).

Auxotrophic mutants of *Physcomitrella* are already known from the literature; however, these mutants were produced by irradiation and chemical mutagenesis of spores (Ashton & Cove 1977; Courtice et al. 1978; Engel 1968; Grimsley et al. 1977) and the identification of the genetic changes might be difficult. In the present paper we present for the first time a detailed study of auxotrophic and albino *Physcomitrella* mutants produced via targeted mutagenesis. The identification of the corresponding genes will be of great importance for applied research (Reski & Frank 2005).

Material and Methods

Mutant production. The production, selection and regeneration of tagged *Physcomitrella* mutants are described in Egener et al. (2002). For shuttle mutagenesis the cDNA was subcloned in the minimal vector pUCMinIV, a derivative of pUC 19. Pooled minimal vectors with target cDNAs were introduced into *E. coli* carrying an inducible transposase (tnpA) and a conjugative plasmid with a derivative of the transposon Tn1000. This transposon carried a nos-promoter driven nptII cassette, which encodes resistance against the antibiotic G418. Induction of the transposase by IPTG resulted in the formation of a cointegrate between the conjugative plasmid and the cDNA clones. Resolution of the cointegrates was achieved by the conjugative transfer into a recipient strain overexpressing the tnpR resolvase gene. This resulted in the release of a copy of the cDNA-carrying minimal vector with an insertion of the mini-Tn1000::nptII. These plasmids were retransformed into *E. coli*.

Two different approaches were used. In the first approach normalized cDNA from an amplified protonema-library were subjected to shuttle mutagenesis and used for transformation (batch transformation). Plants with Bar codes between 1–39815 are batch transformants (including the auxotrophic mutants with Bar Codes 4174, 22564, 36623, 36976, 37989).

In a second approach defined batches of 20 different mutagenized cDNAs were pooled and protoplasts were transformed with these cDNA-mixtures (HoMi-pools). In this second approach cDNAs from three different libraries were used. For the protonema-library, RNA was extracted from moss cultured in liquid medium for different time periods and with different treatments (for example addition of plant growth regulators or abscisic acid). For the gametophore-library fully differentiated moss was harvested from agar plates at different time points, with and without addition of plant growth regulators and glucose. For the sporophyte-library, sporophyte induction was initiated and the sexual organs, spore capsules and spores of *Physcomitrella patens* were harvested at different time points. Additionally regenerating protoplasts were added to the material used for RNA extraction. All libraries were normalized, and the sporophyte- and gametophore-libraries were subtracted cDNA libraries. The two albino plants have been produced by transformation of wildtype

Physcomitrella with defined batches of 20 different cDNAs from the gametophore-library.

Pools of plasmid DNA prepared from transposon-mutagenized cDNAs were used for large-scale PEG-mediated transformation of moss protoplasts grown in liquid culture as described in Hohe et al. (2004). Regeneration was done in liquid regeneration medium for 10 days, followed by three days on solid medium without selection. Afterwards the selection period on antibiotic-containing medium lasted 14 days and a release phase of 14 days on medium without antibiotic and another round of selection followed. Resistant transformants were picked and cultured for another three weeks on supplemented medium without antibiotic.

Plant material and culture conditions. Eleven weeks after transformation each mutant was split into two parts. One copy each was cultured for four weeks on a supplemented medium and eight weeks on minimal medium before the growth ability was determined for 51,180 mutants. In comparison to the standard (wild type plant), mutant growth could be assigned to three categories: (1) good growth like the standard, (2) retarded growth, (3) no growth/very poor growth with the production of only single protonematal filaments.

The minimal medium consists of 1.84 mmol/L KH_2PO_4, 1.02 mmol/L KCl, 3.36 mmol/L $MgSO_4 \times 7H_2O$, 4.24 mmol/L $Ca(NO_3)_2 \times 4H_2O$ and 0.045 mmol/L $FeSO_4 \times 7H_2O$ (modified Knop medium as described in Reski & Abel 1985). The supplemented medium consists of the same macroelements plus 50 g L^{-1} glucose, a vitamine mix (22 µmol/L myo-inositol, 20 µmol/L choline chloride, 8 µmol/L nicotinic acid, 1.5 µmol/L thiamine-HCl, 1.2 µmol/L pyridoxine-HCl, 0.04 µmol/L biotin, 1.8 µmol/L p-aminobenzoic acid, 4.0 µmol/L Ca-D-pantothenate, 0.04 µmol/L riboflavine), 2.5 mmol/L ammonium tartrate, 50 µmol/L adenine, 0.25 g L^{-1} peptone, micro elements (50 µmol/L H_3BO_3, 50 µmol/L $MnSO_4 \times H_2O$, 15 µmol/L $ZnSO_4 \times 7H_2O$, 2.5 µmol/L KJ, 0.5 µmol/L $Na_2MoO_4 \times 2H_2O$, 0.05 µmol/L $CuSO_4 \times 5H_2O$, 0.05 µmol/L $Co(NO_3)_2 \times 6H_2O$), and 13.8 µmol/L Na-palmitic acid. Macro- and microelements, $FeSO_4 \times 7H_2O$ and glucose were autoclaved, the other supplements were filter-sterilized (0.22 µm, Steritop, Millipore) and added to the medium after autoclaving. For all media the pH was adjusted to 5.8 before autoclaving and before filter sterilizing, respectively. Plants were grown *in vitro* at a temperature of 25°C and a light/dark regime of 16/8 h with a light intensity of 70 µmol m^{-2} s^{-1} (Philips TDL 38 W/25).

Because polyploidization is negatively correlated with growth on minimal medium in *Physcomitrella* (Schween et al. 2005b), only haploid mutants were regarded in our study. The ploidy level of plants was checked by flow cytometry. Flow cytometric (FCM) analysis was carried out according to Schween et al. (2003). Suspensions of intact nuclei were prepared from about 5–20 mg of fresh plant material by chopping up the material with a razor blade in a glass petri dish with 1.5 ml of a DAPI-containing buffer (0.01 % DAPI (= 4'6-Diamidino-2-phenyindole), 1.07 g/l $MgCl_2 \times 6\ H_2O$, 5.0 g/l NaCl, 12.11 g/l Tris, 1 ml/l Triton). The solution was filtered through a sieve of 30 µm pore size and fluorescence of stained nuclei was determined with a Partec cell analyser (Partec, Münster, Germany) using a 100 W high-pressure mercury lamp.

Identification of physiological mutants. Wild type plants were used as standard. *Physcomitrella* wildtype was initiated by germinating a single spore from the Hamburg strain, which is a subculture of the strain 16/14 collected in Gransden Wood, Huntingdonshire, U.K. They showed good growth during their culture on minimal medium as well as on the supplemented medium. The screen of 51,180 haploid mutants was performed by comparing the diameter of each mutant to the standard visually.

Analysis of physiological mutants.

Growth-disturbed mutants.—Based on results of the metabolic screen, five auxotrophs, which did not grow on minimal medium, were randomly chosen for further analysis by performance of a medium supplementation test. These mutants were revitalized from cryopreservation according to Schulte and Reski (2004). Their genetic stability after transformation and cryopreservation was verified by a three-week culture on the supplemented medium containing 25 µg/ml G-418 (geneticin). Afterwards, clones of each mutant were cultured on variations of the minimal medium and the supplemented medium, respectively. The minimal medium was enriched with different compounds of the supplemented medium (**Table 1**,

Table 1. Composition of minimal and complete media for complementation tests. The minimal medium (MM, medium no. 1) and the complete medium (CM, medium no. 7) served as controls. Their compositions are described in Materials and Methods. For complementation tests, single compounds of the complete medium were added to the minimal medium (media no. 2–6: MM+compound) or single compounds of the complete medium were lacking (media no. 8–12: CM-compounds).

medium no.	medium composition
1	MM (control 1)
2	MM + glucose
3	MM + vitamin mix
4	MM + ammonium tartrate
5	MM + adenine + peptone
6	MM + micro elements + palmitic acid
7	CM (control 2)
8	CM - glucose
9	CM - vitamin mix
10	CM - ammonium tartrate
11	CM - adenine - peptone
12	CM - micro elements - palmitic acid

media no. 2–6); in contrast, some compounds were absent in the supplemented medium (**Table 1**, media no. 8–12). The clones were cultured in six well-plates. Subcultures were performed every four weeks. Mutant growth was observed for three months.

To identify the defect of a vitamin deficient mutant, a refined test was performed. Clones of this mutant were cultured for four weeks on various minimal media, supplemented with the different components of the vitamin mix. In addition, clones of this mutant were cultured on various supplemented media, lacking single vitamins of the vitamin mix.

Medium supplementation test of albino mutants.—The two albino mutants which were identified in the metabolic screen were analysed by performing the supplementation test as described above.

Albino mutant test of light sensitivity.—Two clones of each albino mutant were cultured for eight weeks on minimal and supplemented medium by using the following light intensities: 5, 10, 30, 50, 70 and 76 µmol m^{-2} s^{-1} (Philips TDL 38 W/25). Plants were subcultured after four weeks.

Results

1) Identification of physiological mutants.

Growth-disturbed mutants.—The growth ability

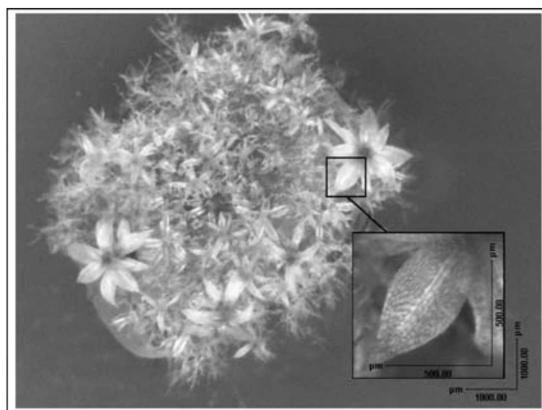

Figure 1. Albino mutant on minimal medium.

of 51,180 haploid Physcomitrella mutants on minimal medium and on supplemented medium was determined. In comparison to the standard (wild type plant), mutant growth could be assigned to three categories: (1) good growth like the standard, (2) retarded growth, (3) no growth/very poor growth with the production of only single protonematal filaments. Pieces of protonema with a diameter of about 3 mm were placed on the medium. On minimal medium the differentiation of gametophores could be observed within the first week and after four weeks the diameter of wildtype colonies was about 1 to 1.5 cm^2. On supplemented medium the plant stayed in the protonematal stage and the diameter increased to about 2 cm. Good growth on both media was observed for most mutants (n=47,915; 93.62%). The growth ability of the remaining mutants (n=3,265; 6.38%) differed from the wild type on at least one medium, indicating different kinds of physiological defects. Mutants without growth on minimal medium but complete rescue of metabolic defects on the supplemented medium were clearly identified as auxotrophic mutants (n=20; 0.04%). A further 31 mutants (0.06%) did not grow on minimal medium but showed also little growth on the supplemented medium.

Albino mutants.—Within the collection of 51,180 haploid mutants, two albino mutants were observed on minimal medium. On the supplemented medium they both were phenotypically identical to the standard. They started to bleach after their transfer to minimal medium and became nearly echlorophyllose except for some green spots (**Fig. 1**).

The retransfer of white plants to supplemented medium caused in turn the production of green protonemata. These two noticeable physiological mutants were analysed further to detect a possible light sensitivity.

2) Analysis of physiological mutants.

Auxotrophic mutants: Performance of a medium supplementation test.—Medium supplementation tests were performed with five randomly chosen auxotrophs (mutants with Bar Codes 4174, 22564, 36623, 36976, 37989). Mutant with Bar Code 22564 survived on minimal medium which was enriched with the vitamin mix (**Table 1**, medium no. 3; **Fig. 2**). In turn it died on the supplemented medium lacking the vitamin mix (**Table 1**, medium no. 9; **Fig. 3**). Culture of this mutant on different minimal media enriched with single vitamins resulted in the unequivocal identification of a p-aminobenzoic auxotroph. No survival was observed on minimal media supplemented with vitamins other than p-aminobenzoic acid. The phenotype and growth of the restored mutant were wildtype-like.

Three mutants (Bar Codes 36623, 36976, 37989) grew well only on a minimal medium, which was supplemented with ammonium tartrate (**Table 1**, medium no. 4). One mutant (Bar Code 37989) also grew well on a minimal medium supplemented with peptone, containing a mixture of amino acids (**Table 1**, medium no. 5). In contrast, very poor growth was observed on each of the other tested minimal media containing nitrate as sole nitrogen source (**Table 1**, media no. 1–3, 6). It can be concluded that the reason for the observed growth properties is a nitrate assimilation deficiency.

One mutant (Bar Code 4174) showed a more complex behaviour. Independent of the supplemented minimal medium (**Table 1**, media no. 2–6), the plant did not grow. On the other hand, it always grew well on the supplemented medium, whatever substance was missing (**Table 1**, media no. 8–12).

A common feature of all auxotrophs was their ability to survive on minimal medium for 3–5 weeks. This observation indicates the importance to observe Physcomitrella mutants for a certain time period to identify the auxotrophic nature unambiguously.

Albino mutants: Performance of medium supplementation tests and test of light sensitivity.—For both albino mutants no deficiency compensation was achieved by their culture on different minimal media, each providing single components of the supplemented medium (**Table 1**, media no. 2–6). The bleaching of mutants was not prevented, independent from the supplementation of the minimal medium. Additionally, both mutants grew well and did not bleach on the supplemented medium, no matter which component was lacking (**Table 1**, media no. 8–12). Different light intensities for plant illumination had also no influence on the bleaching procedure. Both plants bleached during their culture on minimal medium, independently of the light intensity used (5 to 76 μmol m^{-2} s^{-1}). In contrast, the culture of mutants on the supplemented medium always resulted in green plants.

Discussion

The present study was designed to identify haploid *Physcomitrella* knockout mutants disturbed in metabolic pathways. All *Physcomitrella* auxotrophs identified to date have been obtained following plant irradiation or chemical mutagenesis (Ashton & Cove 1977; Courtice et al. 1978; Engel 1968; Grimsley et al. 1977) and so far the responsible genes could not be cloned from these mutants. In contrast, the identification of the relevant genes in the tagged knockout mutants should be possible. Previous Southern analysis of 55 batch transformants showed that on average 10 copies of the npt2-cassette were integrated and that most of the transforming DNA molecules were co-integrated as concatemers in only few loci (Egener et al. 2002). Future use of these knockout mutants in further metabolic studies and identification of the responsible genes will open the perspective of genetic engineering of crop plants with "novel" genes (Reski & Frank 2005). No albino mutants at all have been described for *Physcomitrella* until now.

The present study showed that the performance of an efficient high throughput metabolic screen of *Physcomitrella* knockout mutants resulted in the clear identification of different auxotrophic mutants. As a lower land plant the moss tissue can be easily divided in different pieces of the same size and subsequently cultured on different enriched media for mutant selection. In contrast, mutant identification of the more complex seed plant *Arabisopsis* is rather tedious. Some mutants, and therefore important genetic

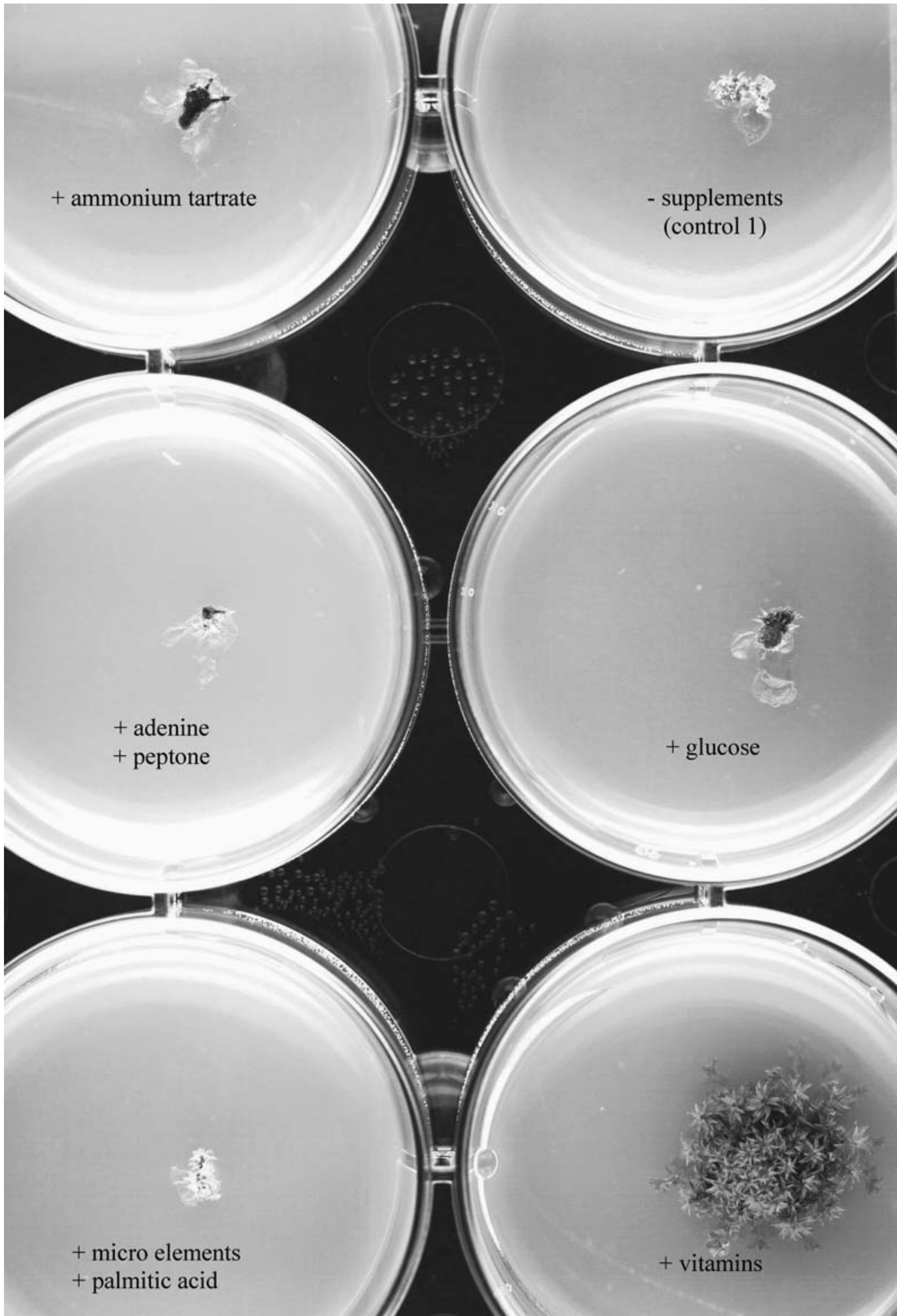

Figure 2. Growth of the vitamin deficient mutant on minimal medium supplemented ("+") with the different compounds of the complete medium.

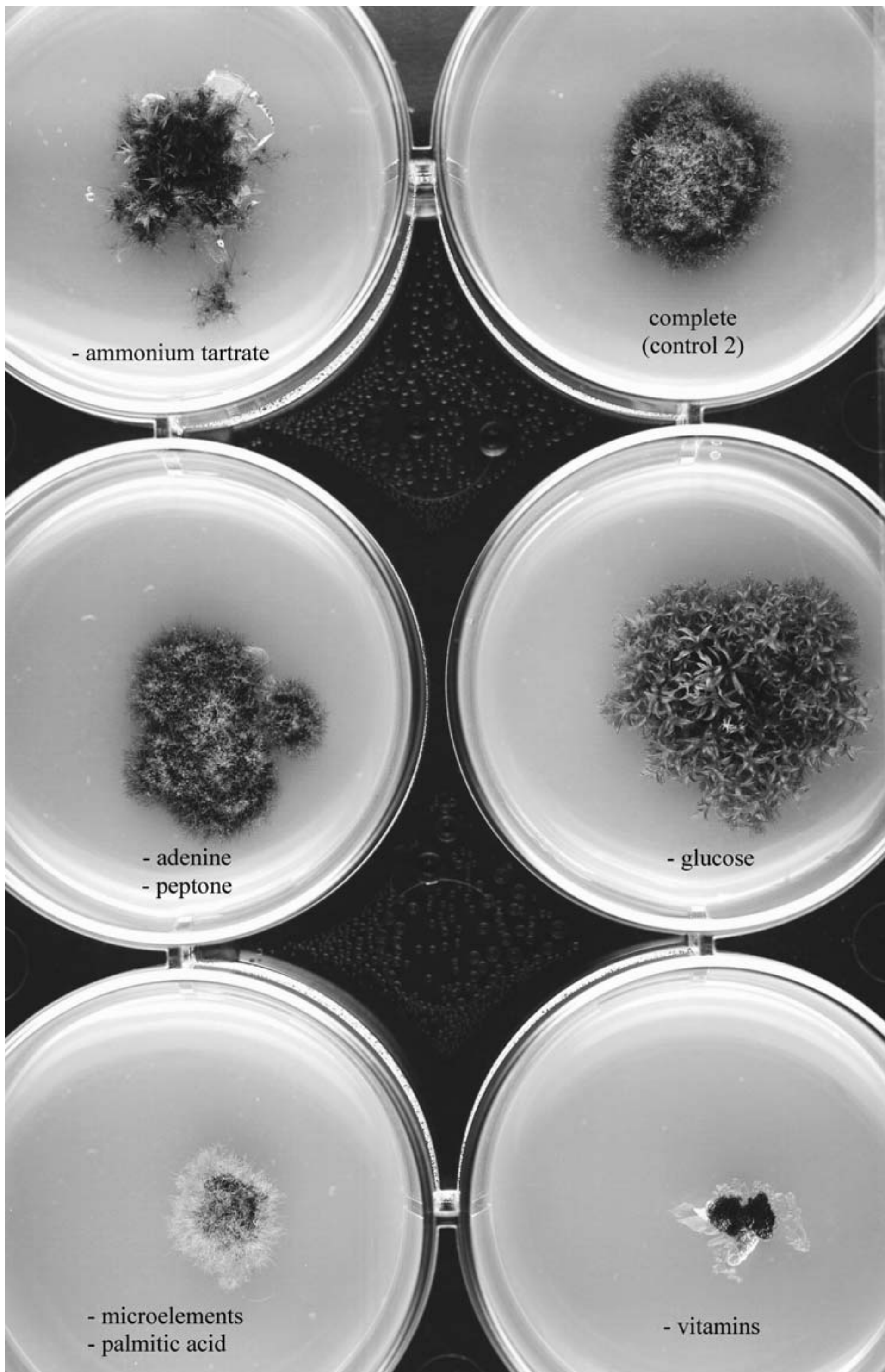

Figure 3. Growth of the vitamin deficient mutant on different complete media. ("complete": complete medium as described in material and methods. "-": Complete medium without the named compounds. The different phenotypes are due to the different media compositions).

information, might be lost. There are hints for a limited identification of *Arabidopsis* auxotrophs due to a widespread redundancy of essential genes and biochemical pathways (Patton et al. 1998). Because the average gene family size in *Physcomitrella* is smaller than in *Arabidopsis* (Rensing et al. 2002) less redundancy might be expected. Additionally, *Physcomitrella* provides a rich source of novel genes (Reski 2003). We conclude that the analysis of *Physcomitrella* metabolic knockouts will not only contribute to existing knowledge gained from *Arabidopsis* and other seed plants. The discovery of completely new genes is also expected.

The present study resulted in the identification of different sorts of growth-disturbed mutants. Auxotrophic mutants were detected due to their missing growth on minimal medium, but were rescued on supplemented medium. Mutants with retarded growth on both media can also be considered to be auxotrophs, since the supplemented medium cannot supply each possible component which might be essential for restoring mutant phenotypes. However, a failed rescue of those mutants could also be ascribed to defective metabolic systems, preventing transport and utilization of components which are present in the medium. The observation of good mutant growth on minimal medium, but retarded growth on supplemented medium may be explained by toxic effects. Uptake of medium compounds might result in toxic metabolite accumulation in transformants, which have defects in key enzymes.

Apart from growth-disturbed mutants, two further noticeable physiological mutants were identified in the present study, consisting of white tissue. No albino mutants have been described until now for *Physcomitrella*. Different reasons are known for the appearance of white plant cells, e.g., a blockage at various steps of the biosynthetic pathway of chlorophyll (Runge et al. 1995; Wang et al. 1996) or an arrest of chloroplast development (Bae et al. 2001). For *Arabidopsis*, albino mutants or albino sectors of variegated phenotypes were used to study gene functions involved in chloroplast development (Carol et al. 1999; Motohashi et al. 2001). The study of albino cotyledons resulted in the isolation and characterization of a cotyledon-specific albino locus (Yamamoto et al. 2000). However, it is known that the study of photosynthetic mutants in plants has been limited, since many defects in photosynthesis are lethal (Heck et al. 1999). *Physcomitrella* albino knockout mutants could provide valuable material to investigate photosynthesis genes, especially due to the high degree of conserved genes between mosses and seed plants (Reski 2003).

In the present study we showed that different light intensities did not affect the bleaching procedure. The appearance of white cells is therefore not caused by photo-oxidation, i.e., by a sensitivity to light stress, as was described for albino mutants of *Arabidopsis* (Hutin et al. 2003). The chlorophyll production in both albino mutants was dependent on the medium composition. We presumed that for both mutants the regulation of gene expression involved in photosynthesis was affected by the availability of medium nutrients. Such regulatory activities by carbon metabolites were described for *Arabidopsis* mutants (Pego et al. 2000). However, the defects in both *Physcomitrella* mutants were not compensated by a defined supplement in the medium. These results indicate a disruption of a complex network of interactions related to photosynthesis in the moss. Although progress in the elucidation of photosynthesis pathways has been rapid since the discovery that several environmental signals are in some instances integrated, translated and perceived by the plant (Pego et al. 2000), the regulatory mechanisms are still poorly understood. Both albino mutants might contribute to the study of the precise mechanisms that regulates photosynthesis. One interesting application would be the use of identified genes as potential targets for new herbicides.

This is the first detailed study of auxotrophic and albino *Physcomitrella* mutants produced by transformation-mediated gene disruption. The mutants will enable the identification of essential and non-redundant genes during plant growth and development. In addition to their suitability for metabolic studies, the albino mutants as well as the auxotrophs can be used for marker-assisted selection and genetic engineering, fuelling plant biotechnology with a rich source of genes, hitherto not known from seed plants (Lang et al. 2005; Reski & Frank 2005).

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Literature Cited

Ashton, N. W. & D. J. Cove. 1977. The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants of the moss *Physcomitrella patens*. Molecular and General Genetics 154: 87–95.

Bae, C., T. Abe, T. Matsuyama, N. Fukunishi, N. Nagata, T. Nakano, Y. Kaneko, K. Miyoshi, H. Matsushima & S. Yoshida. 2001. Regulation of chloroplast gene expression is affected in ali, a novel tobacco albino mutant. Annals of Botany 88: 545–553.

Carol, P., D. Stevenson, C. Bisanz, J. Breitenbach, G. Sandmann, R. Mache, G. Coupland & M. Kuntz. 1999. Mutations in the *Arabidopsis* gene *IMMUTANS* cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. Plant Cell 11: 57–68.

Courtice, G. R. M., N. W. Ashton & D. J. Cove. 1978. Evidence for the restricted passage of metabolites into the sporophyte of the moss *Physcomitrella patens* (Hedw.). Journal of Bryology 10: 191–198.

Egener, T., J. Granado, M. C. Guitton, A. Hohe, H. Holtorf, J. M. Lucht, S. Rensing, K. Schlink, J. Schulte, G. Schween, S. Zimmermann, B. Rak & R. Reski. 2002. High frequency of phenotypic deviations in *Physcomitrella patens* plants transformed with a gene-disruption library. BMC Plant Biology 2: 6.

Engel, P. P. 1968. The induction of biochemical and morphological mutants in the moss *Physcomitrella patens*. American Journal of Botany 55: 438–446.

Girke, T., H. Schmidt, U. Zähringer, R. Reski & E. Heinz. 1998. Identification of a novel delta 6-acyl group desaturase by targeted gene disruption in *Physcomitrella patens*. Plant Journal 15: 39–48.

Girod, P. A., H. Fu, J. P. Zrÿd & R. D. Viestra. 1999. Multiubiquitin chain binding subunit MCB1 (RPN10) of the 26S proteasome is essential for developmental progression in *Physcomitrella patens*. Plant Cell 11: 1457–1472.

Grimsley, N. H., N. W. Ashton & D. J. Cove. 1977. Complementation analysis of auxotrophic mutants of the moss *Physcomitrella patens*, using protoplast fusion. Molecular and General Genetics, 155: 103–107.

Heck, D. A., D. Miles & P. R. Chitnis. 1999. Characterization of two photosynthetic mutants of maize. Plant Physiology 120: 1129–1136.

Hohe, A., T. Egener, J. Lucht, H. Holtorf, C. Reinhard, G. Schween & R. Reski. 2004. An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene knockouts in a moss, *Physcomitrella patens*. Current Genetics 44: 339–347.

Holtorf, H., M. C. Guitton & R. Reski. 2002. Plant functional genomics. Naturwissenschaften 89: 235–249.

Hutin, C., L. Nussaume, N. Moise, I. Moya, K. Kloppstech & M. Havaux. 2003. Early light-induced proteins protect *Arabidopsis* from photooxidative stress. Proceedings of the National Academy of Sciences U.S.A. 100: 4921–4926.

Lang, D., J. Eisinger, R. Reski & S. A. Rensing. 2005. Representation and high-quality annotation of the *Physcomitrella patens* transcriptome demonstrates a high proportion of proteins involved in metabolism in mosses. Plant Biology 7: 238–250.

Motohashi, R., N. Nagata, T. Ito, S. Takahashi, T. Hobo, S. Yoshida & K. Shinozaki. 2001. An essential role of a *Tat*C homologue of a ΔpH-dependent protein transporter in thylakoid membrane formation during chloroplast development in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences U.S.A. 98: 10499–10504.

Nishiyama, T., Y. Hiwatashi, I. Sakakibara, M. Kato & M. Hasebe. 2000. Tagged mutagenesis and gene-trap in the moss, *Physcomitrella patens* by shuttle mutagenesis. DNA Research 7: 9–17.

Patton, D. A., A. L. Schetter, L. H. Franzmann, K. Nelson, E. R. Ward & D. W. Meinke. 1998. An embryo-defective mutant of *Arabidopsis* disrupted in the final step of biotin synthesis. Plant Physiology 116: 935–946.

Pego, J. V., A. J. Kortstee, C. Huijser & S. C. M. Smeekens. 2000. Photosynthesis, sugars and the regulation of gene expression. Journal of Experimental Botany 51: 407–416.

Rensing, S. A., D. Fritzowsky, D. Lang & R. Reski. 2005. Protein encoding genes in an ancient plant: analysis of codon usage, retained genes and splice sites in a moss, *Physcomitrella patens*. BMC Genomics 6: 43.

Rensing, S. A., S. Rombauts, Y. van de Peer & R. Reski. 2002. Moss transcriptome and beyond. Trends Plant Science 7: 535–538.

Reski, R. 1998. *Physcomitrella* and *Arabidopsis*: the David and Goliath of reverse genetics. Trends in Plant Science 3: 209–210.

———. 1999. Molecular genetics of *Physcomitrella*. Planta 208: 301–309.

———. 2003. *Physcomitrella patens* as a novel tool for plant functional genomics. Pages 205–209. *In* I. K. Vasil (ed.), Plant Biotechnology 2002 and Beyond. Kluwer Academic Publishers, Dordrecht.

——— & W. O. Abel. 1985. Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. Planta 165: 354–358.

——— & W. Frank. 2005. Moss (*Physcomitrella patens*) functional genomics—Gene discovery and tool development with implications for crop plants and human health. Briefings in Functional Genomics & Proteomics 4: 48–57.

Runge, S., B. Van Cleve, N. Lebedev, G. M. Armstrong & K.

Apel. 1995. Isolation and classification of chlorophyll-deficient xantha mutants of *Arabidopsis thaliana*. Planta 197: 490–500.

Schaefer, D. G. 2001. Gene targeting in *Physcomitrella patens*. Current Opinion in Plant Biology 4: 143–150.

———& & J. P. Zrÿd. 1997. Efficient gene targeting in the moss *Physcomitrella patens*. Plant Journal 11: 1195–1206.

Schulte, J. & R. Reski. 2004. High throughput cryopreservation of 140.000 *Physcomitrella patens* mutants. Plant Biology 6: 1–9.

Schween, G., T. Egener, D. Fritzkowsky, J. Granado, M.-C. Guitton, N. Hartmann, A. Hohe, H. Holtorf, D. Lang, J. M. Lucht, C. Reinhard, S. A. Rensing, K. Schlink, J. Schulte & R. Reski. 2005a. Large-scale analysis of 73,329 *Physcomitrella* plants transformed with different gene disruption libraries: Production parameters and mutant phenotypes. Plant Biology 7: 228–237.

———, G. Gorr, A. Hohe & R. Reski. 2003. Unique tissue-specific cell cycle in *Physcomitrella*. Plant Biology 5: 50–58.

———, A. Hohe, J. Schulte & R. Reski. 2005b. Effect of ploidy level on growth, differentiation and morphology in *Physcomitrella patens.* The Bryologist 108: 27–35.

Strepp, R., S. Scholz, S. Kruse, V. Speth & R. Reski. 1998. Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein *FtsZ*, an ancestral tubulin. Proceedings of the National Academy of Science U.S.A. 95: 4368–4373.

Thorneycroft, D., S. M. Sherson & S. M. Smith. 2001. Using gene knockouts to investigate plant metabolism. Journal of Experimental Botany 52: 1593–1601.

Wang, B., A. Guo & P. Wang. 1996. Changes of chlorophyll metabolism during the albinic stage of a wheat mutant. Acta Botanica Sinica 38: 557–562.

Yamamoto, Y. Y., P. Puente & X. W. Deng. 2000. An *Arabidopsis* cotyledon-specific albino locus: a possible role in 16S rRNA maturation. Plant Cell Physiology 41: 68–76.

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