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Methodology article

High frequency of phenotypic deviations in *Physcomitrella patens* plants transformed with a gene-disruption library

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Abstract

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

Results: As an important prerequisite for a large-scale gene/function correlation study in this plant, we are establishing a collection of *Physcomitrella patens* transformants with insertion mutations in most expressed genes. A low-redundancy moss cDNA library was mutagenised in *E. coli* using a derivative of the transposon Tn*1000*. The resulting gene-disruption library was then used to transform Physcomitrella. Homologous recombination of the mutagenised cDNA with genomic coding sequences is expected to target insertion events preferentially to expressed genes. An immediate phenotypic analysis of transformants is made possible by the predominance of the haploid gametophytic state in the life cycle of the moss. Among the first 16,203 transformants analysed so far, we observed 2636 plants (= 16.2%) that differed from the wild-type in a variety of developmental, morphological and physiological characteristics.

Conclusions: The high proportion of phenotypic deviations and the wide range of abnormalities observed among the transformants suggests that mutagenesis by gene-disruption library transformation is a useful strategy to establish a highly diverse population of *Physcomitrella patens* mutants for functional genome analysis.

Background

The most informative approach to identify a function for a given gene is the precise inactivation or functional alteration of the gene, followed by the analysis of the phenotypic change resulting from this manipulation. Gene targeting based on homologous recombination between a targeting construct with altered or abolished gene function and its cognate endogenous gene has been a highly successful approach for gene function analysis in prokaryotes, lower eukaryotes, and mice. Unfortunately, in higher plants this approach is restricted by the very low ratio of 10^{-3} to 10^{-5} targeted relative to illegitimate recombination events. Although a few homologous recombination events between incoming targeting constructs and their cognate genomic sequences have been described, homologous recombination remains very inefficient and gene targeting thus is not routinely possible in higher plants [1,2]. In contrast, gene targeting via homologous recombination occurs with high frequency in the moss *Physcomitrella patens*[3,4]. After the first demonstration of high-frequency recombination between chromosomal sequences and homologous DNA introduced by transformation [5], gene targeting in Physcomitrella was used successfully to study the function of several genes by creating functional knockouts [6–9]. The high specificity provided by homologous recombination even allows the specific targeting of single members of multi-gene families [10].

The ease of its genetic manipulation, together with a high degree of conservation of morphological features, biochemical pathways and signal transduction mechanisms between *Physcomitrella patens* and higher plants [11–14] has made the moss an important model system for plant functional genomics. To facilitate a large-scale study of plant gene function using *Physcomitrella patens* as a model organism, we are developing a collection of Physcomitrella plants with insertion mutations that affect a wide variety of developmental, morphological and physiological characteristics. Transformation with constructs carrying sequences homologous to the genome typically results in 10-fold higher transformation frequencies then the use of non-homologous constructs, and among these transformants a high proportion shows integration of the construct at the homologous genomic locus [3,12]. We argued that – compared to a random mutagenesis strategy [15] – targeting insertion mutations towards expressed genes would increase the proportion of transformants displaying altered properties, and would decrease the total number of transformants to be screened to find a particular change in phenotype. We therefore developed an efficient transposon-based shuttle mutagenesis system for moss cDNA libraries, and have used pools of insertion-mutagenised cDNA clones tagged with a *nos*-regulated *nptII* selection cassette for the transformation of Physcomitrella plants (Fig. 1).
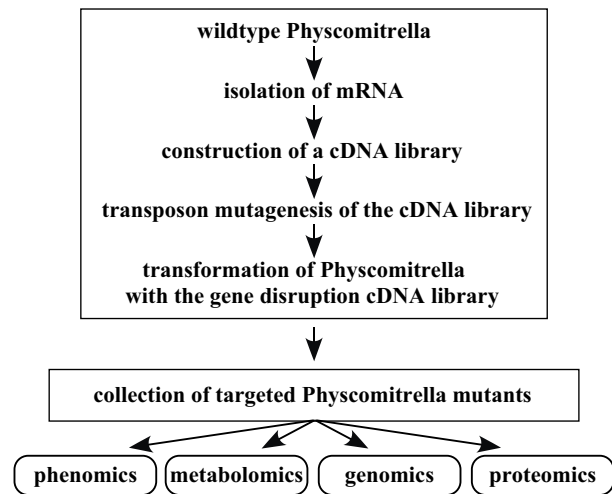

Figure 1
Flow-scheme for the establishment of a Physcomitrella gene disruption mutant collection.

Results and Discussion

cDNA library

To establish a Physcomitrella cDNA library representing most genes expressed during vegetative growth before the onset of differentiation, RNA was extracted from protonemata cultured for different time periods in liquid culture, and a cDNA library in plasmid vectors was established after normalization to decrease redundancy [16]. Mass DNA sequencing and clustering of 57,000 EST sequences yielded 12,000 non-overlapping sequence clusters, and showed a low degree of clone redundancy in the cDNA library used. Sequence analysis of these contigs, together with a large number of additional EST sequences derived from other growth stages and tissues, suggest that the total number of coding sequences for the moss *Physcomitrella patens* and the flowering plant *Arabidopsis thaliana* is similar (Rensing et al., submitted), despite a three-fold larger genome size for the moss [12].

Gene-disruption library

To create a gene-disruption library of cDNA clones carrying insertion mutations, cDNA clones were subjected to shuttle mutagenesis in E. coli. First, the normalised cDNA pool from the amplified protonema library was subcloned into the minimal vector pUCMinIV (Fig. 2). This plasmid is a 1.7 kb derivative of pUC19 from which we have deleted most non-essential DNA sequences to remove insertion targets within the vector sequence. Pooled minimal vectors with target cDNAs were introduced into a donor E. coli strain carrying an inducible transposase gene (tnpA) and a conjugative plasmid with a derivative of transposon Tn1000. This mini-transposon carries a
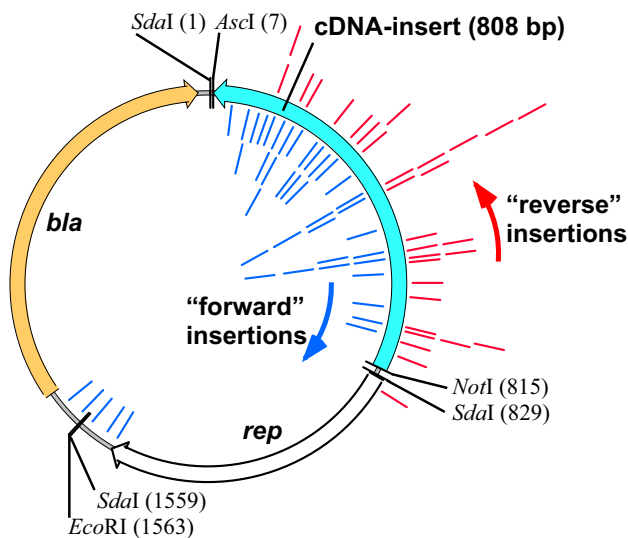
Figure 2
Shuttle transposon mutagenesis of *Physcomitrella patens* cDNA clones. The structure of a representative moss cDNA clone (ID: S_PP015059353; 808 bp) in the pUC-MinIV minimal vector is shown. This defined plasmid was subjected to shuttle mutagenesis, and the transposon insertion sites for 72 resulting clones were mapped by DNA sequencing to assess the distribution of insertions. 41 insertions in "forward" orientation (*nptII* resistance marker on transposon and *bla* marker on vector transcribed in same orientation) are indicated by blue lines within the circle, 31 "reverse" insertions by red lines outside. Most of the insertions (66 / 72, corresponding to 92%) occurred throughout the cDNA, without apparent strong bias for insertion site or orientation. For production of the gene-disruption library used afterwards for the moss transformation, cDNA clones were mutagenised in pools; here about 70% of the mutagenised plasmids had insertions in the cDNA.

Figure 3
Isolation of metabolic mutants. Physcomitrella plants derived from transformation with the gene-disruption library and regenerated on supplemented medium were split in two parts, which were transferred to minimal Knop medium with and without supplements (see Materials) and cultured for 8 weeks. The picture shows four independent Physcomitrella transformants cultured on minimal medium, one of which (bottom right) displays a clear growth defect. All four plants grew equally well on supplemented medium (data not shown). The scale bar at the top indicates size in centimetres.

modified nos-promoter driven nptII expression cassette encoding resistance against the antibiotic G418 as selectable plant marker gene between the border repeat sequences of Tn1000 required for transposition [17]. Induction of transposase activity by IPTG results in transposition and the formation of a cointegrate between conjugative plasmid and cDNA clone. Resolution of the cointegrates was achieved by conjugative transfer into a recipient strain overexpressing the tnpR resolvase gene, resulting in the release of a copy of the cDNA-carrying minimal vector with an insertion of the mini-Tn1000::nptII. These plasmids were isolated and retransformed into E. coli. Analysis of individual clones from this gene-disruption library, as well as model experiments using defined cDNA clone targets showed that in most of the resulting tagged plasmids the transposon had inserted into the moss cDNA, with no apparent preference for target sequence or insertion orientation (Fig. 2).
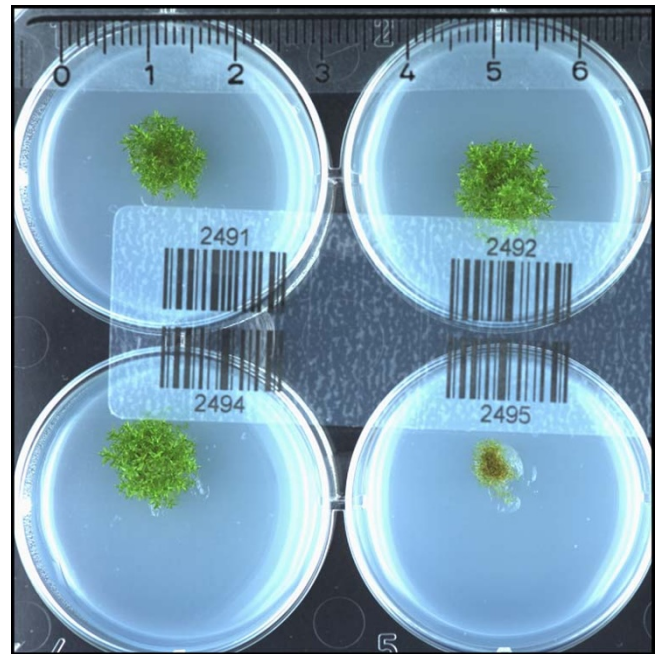
Transformation
Pools of plasmid DNA prepared from transposon-mutagenised cDNAs were used for large-scale PEG-mediated transformation of moss protoplasts grown in semi-continuous bioreactor cultures [6,18,19]. Before transformation, the plasmid DNA was linearised by digestion with a rare-cutting restriction enzyme, *Sda*I, that cuts in the minimal vector just outside of the cDNA inserts (Fig. 2). Regeneration of protoplasts was done on supplemented Knop medium for 2 weeks without selection, followed by two cycles of G418 selection to eliminate unstable transformants [20]. More than 98% of surviving plants were stable transformants carrying the *nptII* selection cassette used for the cDNA disruption, as demonstrated by PCR-detection of the *nptII* coding sequence or a third selection step [21]. The cellular DNA content of all transformants was checked by flow cytometry [18]; 7.7% of the transformants tested (1242 of 16203) were polyploid. Currently, we have produced more than 22,000 moss transformants; the current production capacity is about 3,000 new trans-
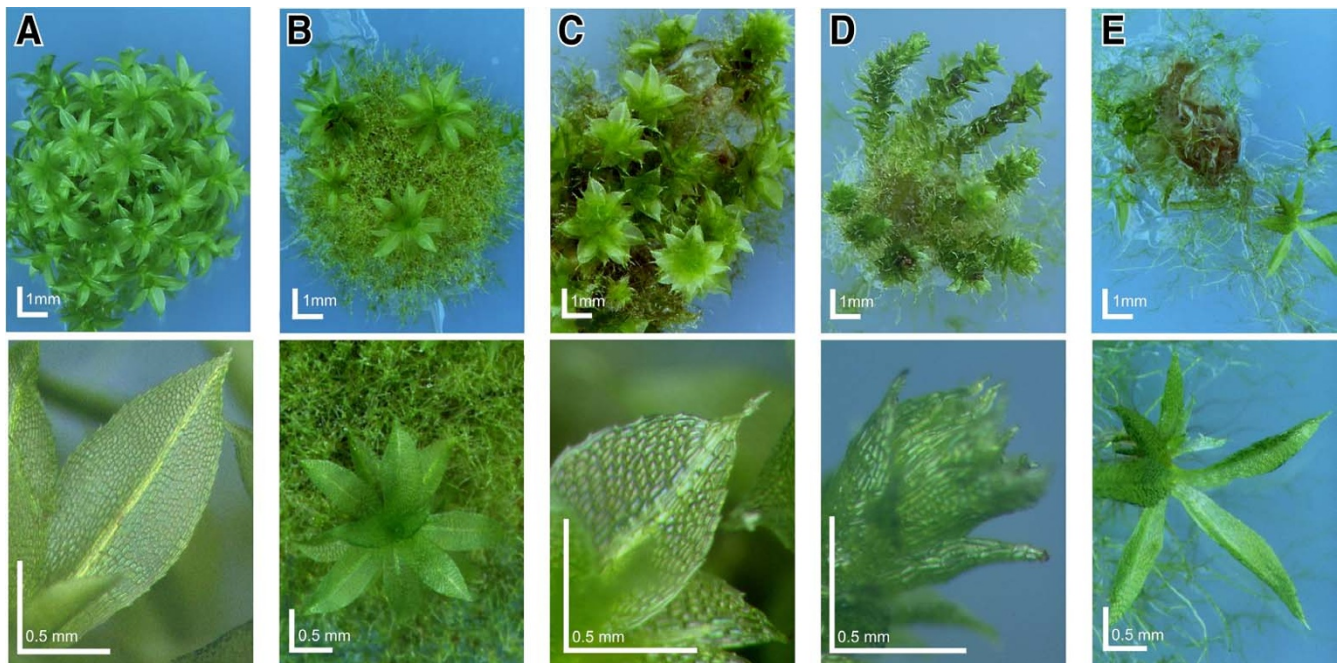
Figure 4
Deviating phenotypes induced in gene-disruption library transformants. Physcomitrella wild-type and transformed plants were grown on minimal Knop medium to induce differentiation and development of gametophores. For each plant, an overview (upper row, scale bar corresponds to 1 mm) and a close-up (bottom row, scale bar equals 0.5 mm) is shown. A, Haploid wild-type moss plant completely covered with leafy gametophores and close-up of wild-type leaf. B, Transformant BC22189 affected in differentiation, mostly comprising of filamentous protonema with reduced number of gametophores, but normal leaf morphology (haploid). C, Transformant BC11280 showing retarded growth, a reduced number of gametophores per moss plant and altered leaf morphology ("drehzipfel" phenotype; twisted tips of leaves, haploid). D, Transformant BC1015 displaying altered growth habitus ("wasserpest" phenotype, reminiscent of the waterweed *Elodea*) and altered leaf morphology (polyploid). E, Transformant BC22288 showing retarded growth and elongated, narrow leafs (polyploid).

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formants per month. This will allow us to establish a large collection of Physcomitrella plants transformed with gene-disruption library constructs; our aim is to obtain a collection of plants carrying mutations in the majority of expressed moss genes.

Physiological mutants

To test for physiological alterations induced in moss plants after transformation with the gene-disruption library, we looked for changed nutritional requirements induced in the transformants (Fig. 3). 7.2% (1,163 of 16,203) of the regenerated plants showed a retarded growth during the culture on minimal medium but a normal growth during the culture on the same medium containing a combination of supplements (see Materials), whereas wild-type plants grew well on both media. This suggests a metabolic defect in transformants with a slow-growth phenotype on minimal medium that could be rescued by supplying metabolites in the medium. 3.1% (508 of 16,203) of the transformants showed a retarded growth during the culture on minimal medium as well as on supplemented medium. Those plants might require substanc-

es that are not present in our supplemented medium, or they might be defective in another way. Different nutritional mutants of Physcomitrella patens, e.g. resulting from mutagenised spores [22] have been described previously.

Morphological mutants

To screen for morphological and developmental mutations, transformants were microscopically observed after regeneration and selection on supplemented Knop medium for 11 weeks followed by growth on minimal Knop medium for 8 weeks. Under these conditions, the filamentous structure (protonema) that is initially formed by regenerating wild-type protoplasts undergoes a developmental switch. It forms a three-faced apical meristem bud, and differentiates into leafy stems, the gametophores (Fig. 4A), that will eventually carry the sex organs. 16.2% (2,631 of 16,203) of the gene-disruption library transformants showed morphological deviations in one or more of the features observed: structure and colour of the moss plant, coverage of the plant by gametophores, shape and uniformity of leaves and arrangement of cells
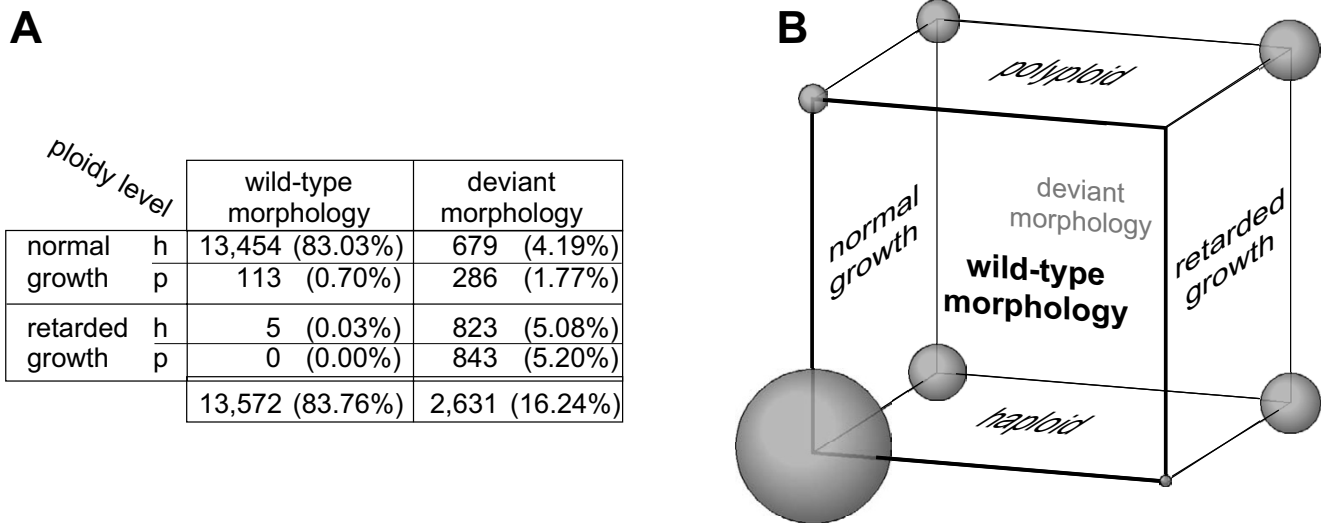
A

ploidy level		wild-type morphology	deviant morphology
normal growth	h	13,454 (83.03%)	679 (4.19%)
	p	113 (0.70%)	286 (1.77%)
retarded growth	h	5 (0.03%)	823 (5.08%)
	p	0 (0.00%)	843 (5.20%)
		13,572 (83.76%)	2,631 (16.24%)

B

Figure 5
Combinations of characteristics observed in Physcomitrella knock-out library transformants. A, 16,203 Physcomitrella plants resulting from transformation with the gene-knockout library were assessed for morphological deviations, retarded growth on minimal medium, and ploidy level [18]. Absolute numbers as well as relative percentages for each of the eight possible combinations of attributes are shown in the table. B, Graphic representation of the observed combinations of attributes. The three characteristics assayed for each transformant are represented by the three dimensions of a cube. Spheres at the corners of the cube denote the eight possible combinations of characteristics; their volume is proportional to the number of transformants observed for each class.

within the leaves (Table 1). Such deviations were not observed to occur spontaneously in 350 analysed wild-type plants and in less than 1% of 400 plants derived from protoplasts mock-transformed without DNA and regenerated in the absence of antibiotic. Examples for deviating phenotypes observed in gene-disruption library transformants are shown in Fig. 4b to 4E. In addition to the phenotypic classes described above, we observed other developmental abnormalities, like the formation of outgrowths on the leaf surface, the formation of thread-like appendages on leaf tips, and an increased number of dark-coloured sectors on leaves. Therefore, a wide spectrum of morphological and developmental alterations is observed in moss plants transformed with a gene-disruption library. In addition, between the three parameters we assayed for each transformant – growth requirements, morphology and ploidy level – there did not appear to be a strict correlation, and we found various combinations of characteristics (Fig. 5).

Molecular analysis
For molecular analysis, genomic DNA was isolated from randomly chosen transformants and analysed in Southern blot experiments. To obtain an estimate for the number of gene-disruption constructs integrated in the transformed moss plants, this DNA was digested with PvuII, which has a single recognition site within the nptII coding sequence.

This results in the formation of two fragments detected by a nptII-derived probe for each copy of the selection cassette. The length of these fragments differs depending on the adjacent sequences, which can be either derived from genomic sequences or from cDNA contributed by the transforming DNA. On Southern blots of 55 transformants, 19.3 ± 3.6 (mean ± s. d.) bands were detected, suggesting an average insertion number of about 10 nptII-cassettes per transformant (data not shown). To discriminate between independent integration events at different genomic positions and co-integrations at the same locus, genomic DNA of 16 transformants was digested with several restriction enzymes that cut genomic Physcomitrella DNA frequently but do not cut within the nptII cassette, and only rarely within the cDNA sequences carried by the transforming DNA (Fig. 6). Most of the genomic DNA was cut by these enzymes into fragments ranging from 1 to 15 kb, as judged by ethidium-bromide staining after gel electrophoresis. However, the majority of fragments detected after hybridisation with the disruption-construct specific nptII-probe were more than 20 kb in size; only few distinct nptII-hybridising bands were observed. Digestion of the same DNA with PvuII, which cuts within the nptII selection cassette, resulted in several nptII-hybridising fragments of smaller size ranging between 1 and 10 kb (Fig. 6). This suggests that most of the transforming DNA molecules are co-integrated in close vicinity to each other,

Table 1: Phenotypic characterisation of 16,203 gene-disruption library transformants

Deviating characteristic:	percentage of deviating plants[1]
Deviations from wild-type for any morphological characteristic	16.2%
- Plant structure (less compact than wild-type, fluffy)	13.1%
- Color (darker or lighter than wild-type)	8.0%
- Coverage of plant by gametophores (coverage less than 95%; wild-type: completely covered)	8.0%
- Leaf shape (width/length ratio or symmetry different from wild type)	12.3%
- Cell shape (irregular patterns, or cell size different than wild-type)	10.1%
- Uniformity of leaves (more than 10% deviating on one plant, wild-type: all uniform)	6.3%

[1]Transformants were assayed independently for the phenotypic alterations shown, some plants deviated from the wild-type in more than one characteristic. The percentages shown for single characteristics therefore add up to more than the total percentage of deviating plants.

without intervening genomic sequences, at few genomic loci. PCR amplification of transgene sequences between the nptII-insertions using nptII-primers and sequence analysis of the obtained products confirmed tandem integrations of different gene disruption constructs in close vicinity (data not shown). Similar integration patterns of transforming DNA molecules in Physcomitrella have been observed previously [5,15].

Conclusions
Taken together, the collection of Physcomitrella transformants that we are establishing by a cDNA based gene-disruption library transformation approach shows a high degree of phenotypic diversity. The fraction of plants with altered morphological or developmental features – 16.2% – exceeds that which has been previously described for a shuttle mutagenesis strategy using genomic clones of moss DNA (3.9%) [15]. This difference might be caused by preferential, homologous recombination-driven disruption of expressed moss genes after the cDNA mutagenesis approach. The frequency of altered phenotypes in our collection of moss transformants also is considerably higher than the less than 2% reported for confirmed Arabidopsis knockout mutants [23], which might be attributed to a lower degree of gene redundancy in Physcomitrella (Rensing et al., submitted). The small number of genomic loci tagged by insertion of gene-disruption library constructs in plants from our mutant collection should allow the recovery of insertion sites and the analysis of moss genes affected in transformants with altered phenotypes. Indeed, we have re-isolated genomic sequences surrounding *nptII*- insertion sites e. g. by PCR-based methods. The presence of introns in some of these sequences suggests that these were derived from the genomic target locus, into which transgene sequences had inserted by homologous recombination (data not shown). We therefore expect that the collection of moss mutants being established here will help to identify novel genes previously unknown in plants [7,12] and will allow to rapidly link DNA se-

quence and functional information. Given the high degree of genetic and physiological conservation between moss and higher plants, this collection of gene disruption library transformants will be a valuable tool not only for gene function studies in the moss Physcomitrella, but for plant functional genomics in general.

Materials and Methods
Transposon mutagenesis
Plasmid constructions and microbiological techniques followed standard procedures [24,25], details have been described in patent WO 01/38509 and can be obtained under [http://ep.espacenet.com] from the European Patent Office or upon request from us. Briefly, *Physcomitrella patens* cDNA libraries subcloned into the minimalised vector pUCMinIV (encoding ampicillin resistance) were transformed into the donor *E. coli* strain R2117. This strain carries a plasmid-encoded IPTG-inducible transposase gene (*tnpA*), and a conjugative plasmid comprising the transfer region from plasmid R388 [26], a chloramphenicol resistance marker and a *nos*-promoter-*nptII* -*nos* terminator expression cassette flanked by the transposon Tn*1000* border repeat sequences required for transposition [17]. The *nptII* cassette was derived from pBIN19 [27]. To increase the efficiency of subsequent plant selection, a point mutation present in the *nptII* coding sequence [28] was reverted to the wild-type sequence. Ampicillin-resistant R2117 donor strain transformants with moss cDNA clones were pooled, treated with IPTG to induce transposase activity, and cocultivated with R1037 recipient cells in the presence of IPTG. *E. coli* strain R1037 carries an IPTG-inducible *tnpR* resolvase gene and a streptomycin resistance locus, both encoded on the chromosome. Tn*1000* transposition in the donor results in the formation of cointegrates between conjugative plasmid and moss cDNA clones, which then can be transferred by conjugation into the recipient where resolution occurs. Recipient cells with mutagenised moss cDNA clones were
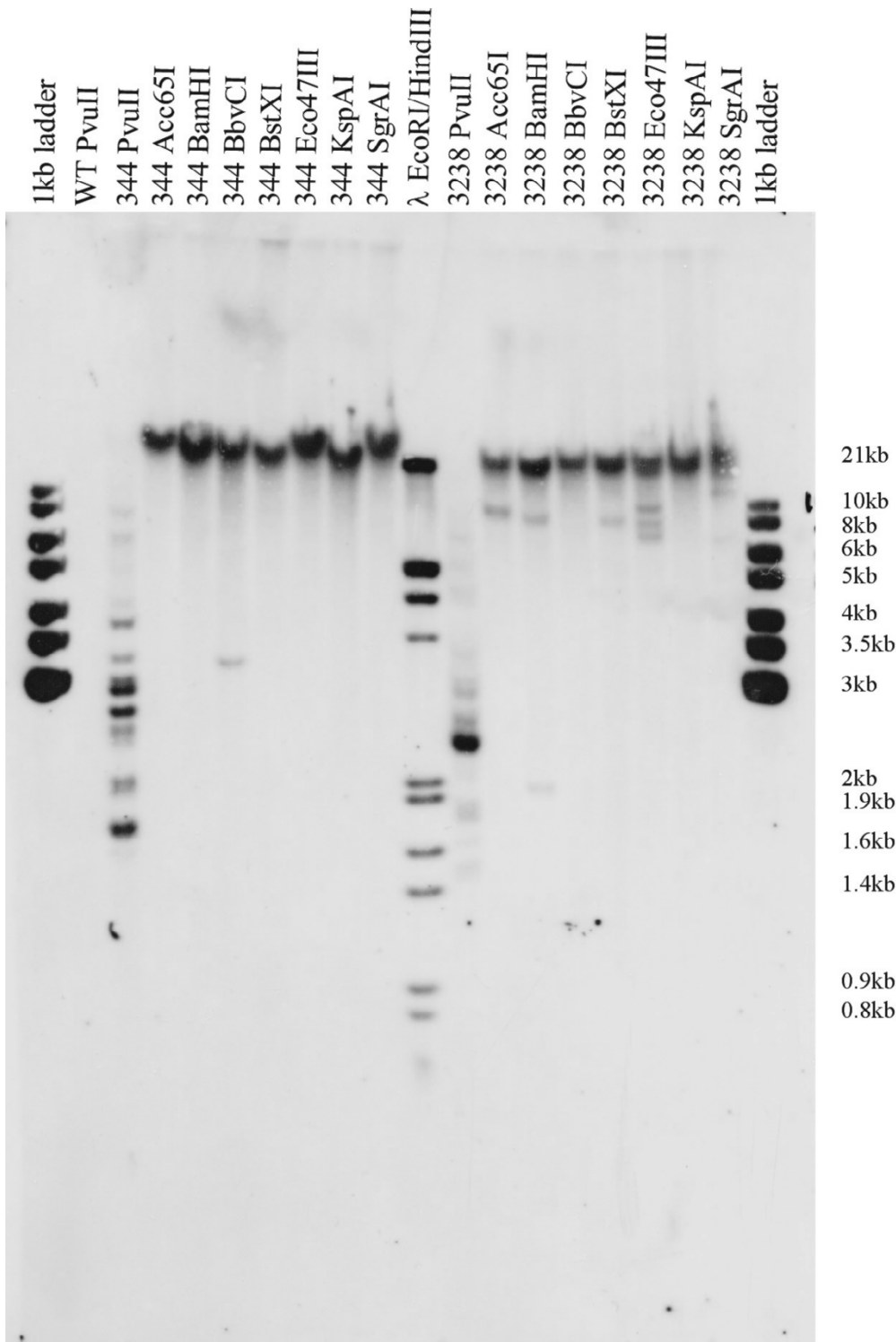
Figure 6
Southern blot analysis of gene-disruption library transformants. I μg of genomic DNA isolated from two representative Physcomitrella transformants (BC344 and BC3238) was digested to completion with *Pvu*II, which has a recognition site within the *nptII* coding sequence, or one of seven other restriction enzymes that do not cut within *nptII* and only rarely in Physcomitrella cDNAs. Fragments carrying *nptII* -sequences were detected by a DIG-labelled probe after electrophoresis and transfer to a nylon membrane.

selected by their simultaneous resistance against ampicillin and streptomycin, and plasmid DNA prepared from pooled cells was used then to transform *P. patens*.

Plant growth conditions, media and transformation

Physcomitrella patens (Hedw.) B.S.G. was cultured in liquid or on solid modified Knop medium as described [29]. For protoplast isolation, protonema was grown in semi-continuous bioreactor cultures supplemented with 2.5 mM ammonium tartrate [18,19]. Transformations [6] were performed with 3×10^5 cells and 50 μg of linearised plasmid DNA. After transformation, plants were grown on Knop medium supplemented with MS-microelements [30], 4 mg/l myo-inositol, 2.8 mg/l choline chloride, 1 mg/l nicotinic acid, 0.5 mg/l thiamine-HCl, 0.25 mg/l pyridoxine, 0.01 mg/l biotin, 0.25 mg/l p-aminobenzoic acid, 1.9 mg/l Ca-D-pantothenate, 0.015 mg/l riboflavine, 6.76 mg/l adenine, 3.84 mg/l Na-palmitinic acid, 250 mg/l peptone, 920 mg/l ammoniumtartrate and 50 g/l glucose, to facilitate survival of metabolic mutants. Stable transformants were identified by a first selection step on 25 μg/ml G418 for 2 weeks, a non-selective release step of 2 weeks, and further 2 weeks of G418 selection.

Molecular analysis

The presence of transgenes in G418 resistant moss transformants was confirmed by PCR with *nptII* specific primers, or a third selection step as described [21]. For Southern blot analyses, 1 μg of genomic DNA isolated by a modified CTAB method [31] was digested for 5–6 hours with 20 U of restriction enzymes purchased from MBI Fermentas or New England Biolabs. After electrophoresis on 0,7% agarose gels the DNA was transferred onto positively charged nylon membrane (Roche). Fragments carrying *nptII* -sequences were detected using hybridisation and blocking solutions as well as Anti-digoxigenin-AP conjugate from Roche but CDP-Star from Promega. The DIG-labelled *nptII* probe was generated by PCR using the random primed labelling mix from Roche and *Taq* polymerase from Promega.

Availability of materials and mutant plants

Materials, single plant lines described in this communication, as well as sequence information for Physcomitrella EST clones with similarity to defined genes of interest will be available for non-profit research after completion of an appropriate material transfer agreement with BASF Plant Science GmbH and Freiburg University. Also, inquiries for sequence information for Physcomitrella EST clones with similarity to defined genes of interest are encouraged. Requests should be directed to the corresponding author (RR).

Authors' contributions

Author 1 (TE) developed cloning strategies for transgene insertion sites. Author 2 (JG) optimised and adapted the transposon mutagenesis procedure for high throughput production. Author 3 (MCG) constructed minimal cloning vectors and was involved in cDNA library production. Author 4 (AH) set up and co-ordinated the high-throughput transformation and mutant production. Author 5 (HH) did molecular analysis of mutagenized cDNA libraries and transgene insertion sites. Author 6 (JML) is submitting author for this communication, composed manuscript and figures, and contributed to the molecular analysis of moss transformants. Author 7 (SAR) was responsible for bioinformatic analysis of cDNA sequences. Author 8 (KS) did molecular characterisation of transformants and the Southern blot analysis. Author 9 (JS) was responsible for the phenotypic documentation, the metabolic screen and the cryoconservation of mutants. Author 10 (GS) was responsible for quality control of transformants by flow cytometry and PCR-detection of transgenes. Author 11 (SZ) developed vectors and the basic methodology for transposon mutagenesis of cDNA clones. Author 12 (ED) is scientific co-ordinator at BASF Plant Science GmbH for the research collaboration with Freiburg University. Author 13 (BR) devised the transposon mutagenesis strategy for the cDNA library and supervised the development of the method. Author 14 (RR) conceived of and leads the Physcomitrella functional genomics project at Freiburg University; he is corresponding author. All authors read and approved the final manuscript.

Acknowledgements

References

1. Mengiste T, Paszkowski J: **Prospects for the precise engineering of plant genomes by homologous recombination.** *Biol. Chem.* 1999, **380**:749-758
2. Puchta H: **Gene replacement by homologous recombination in plants.** *Plant Mol. Biol.* 2002, **48**:173-182
3. Schaefer DG: **Gene targeting in *Physcomitrella patens*.** *Curr. Opin. Plant Biol.* 2001, **4**:143-150
4. Reski R: ***Physcomitrella* and *Arabidopsis*: the David and Goliath of reverse genetics.** *Trends Plant Sci.* 1998, **3**:209-210
5. Schaefer DG, Zryd JP: **Efficient gene targeting in the moss *Physcomitrella patens*.** *Plant J.* 1997, **11**:1195-1206
6. Strepp R, Scholz S, Kruse S, Speth V, Reski R: **Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein FtsZ, an ancestral tubulin.** *Proc. Natl. Acad. Sci. USA* 1998, **95**:4368-4373
7. Girke T, Schmidt H, Zähringer U, Reski R, Heinz E: **Identification of a novel D6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*.** *Plant J.* 1998, **15**:39-48
8. Girod PA, Fu H, Zryd JP, Vierstra RD: **Multiubiquitin chain binding subunit MCB1 (RPN10) of the 26S proteasome is essential for developmental progression in *Physcomitrella patens*.** *Plant Cell* 1999, **11**:1457-1472

9. Imaizumi T, Kadota A, Hasebe M, Wada M: **Cryptochrome Light Signals Control Development to Suppress Auxin Sensitivity in the Moss Physcomitrella patens.** *Plant Cell* 2002, **14:**373-386

10. Hofmann AH, Codon AC, Ivascu C, Russo VE, Knight C, Cove D, Schaefer DG, Chakhparonian M, Zryd JP: **A specific member of the *Cab* multigene family can be efficiently targeted and disrupted in the moss *Physcomitrella patens*.** *Mol. Gen. Genet.* 1999, **261:**92-99

11. Reski R: **Development, genetics and molecular biology of mosses.** *Bot. Acta* 1998, **111:**1-15

12. Reski R: **Molecular genetics of Physcomitrella.** *Planta* 1999, **208:**301-309

13. Wood AJ, Oliver MJ, Cove DJ: **Bryophytes as model systems.** *Bryol.* 2000, **103:**128-133

14. Schaefer DG, Zryd JP: **The moss *Physcomitrella patens*, now and then.** *Plant Physiol.* 2001, **127:**1430-1438

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

16. Ko MS: **An 'equalized cDNA library' by the reassociation of short double- stranded cDNAs.** *Nucl. Acids Res.* 1990, **18:**5705-5711

17. Sherratt DJ: **Tn3 and related transposable elements: site-specific recombination and transposition.** *In: Mobile DNA (Edited by: DE Berg, MM Howe) Washington, D.C: American Society for Microbiology;* 1989, 163-184

18. Hohe A, Schween G, Reski R: **Establishment of a semicontinuous bioreactor culture of *Physcomitrella patens* for mass production of protoplasts.** *Acta Hort.* 2001, **560:**425-428

19. Hohe A, Reski R: **Optimisation of a bioreactor culture of the moss *Physcomitrella patens* for mass production of protoplasts.** *Plant Sci.* 2002

20. Ashton NW, Champagne CEM, Weiler T, Verkoczy LK: **The bryophyte *Physcomitrella patens* replicates extrachromosomal transgenic elements.** *New Phytol.* 2000, **146:**391-402

21. Schween G, Fleig S, Reski R: **High-throughput-PCR screen of 15,000 transgenic Physcomitrella plants.** *Plant Mol. Biol. Rep.* 2002, **20:**43-47

22. Ashton NW, Cove DJ: **The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants in the moss *Physcomitrella patens*.** *Mol. Gen. Genet.* 1977, **154:**87-95

23. Bouche N, Bouchez D: **Arabidopsis gene knockout: phenotypes wanted.** *Curr. Opin. Plant Biol.* 2001, **4:**111-117

24. Sambrook J, Russell D: **Molecular cloning: a laboratory manual (third edition).** *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press;* 2001

25. Miller JH: **A short course in bacterial genetics.** *Cold Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press;* 1992

26. Avila P, de la Cruz F: **Physical and genetic map of the IncW plasmid R388.** *Plasmid* 1988, **20:**155-157

27. Bevan M: **Binary Agrobacterium vectors for plant transformation.** *Nucl. Acids Res.* 1984, **12:**8711-8721

28. Yenofsky RL, Fine M, Pellow JW: **A mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selection pressure.** *Proc. Natl. Acad. Sci. USA* 1990, **87:**3435-3439

29. Reski R, Abel WO: **Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine.** *Planta* 1985, **165:**354-358

30. Murashige T, Skoog F: **A revised medium for rapid growth and bioassays with tobacco tissue culture.** *Physiol. Plant.* 1962, **15:**473-497

31. Reski R, Wehe M, Hadeler B, Marienfeld JR, Abel WO: **Cytokinin and light quality interact at the molecular level in the chloroplast mutant PC22 of the moss Physcomitrella.** *J. Plant Phys.* 1991, **138:**236-243