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Comparison of gene targeting efficiencies in two mosses suggests that it is a conserved feature of Bryophyte transformation

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Abstract The moss, *Physcomitrella patens*, is a novel tool in plant functional genomics due to its exceptionally high gene targeting efficiency that is so far unique for plants. To determine if this high gene targeting efficiency is exclusive to *P. patens* or if it is a common feature to mosses, we estimated gene-targeting efficiency in another moss, *Ceratodon purpureus*. We transformed both mosses with replacement vectors corresponding to the adenine phosphoribosyl transferase (*APT*) reporter gene. We achieved a gene targeting efficiency of 20.8% for *P. patens* and 1.05% for *C. purpureus*. Our findings support the hypothesis that efficient gene targeting could be a general mechanism of Bryophyte transformation.

Keywords Gene targeting · Homologous recombination · Moss · *Physcomitrella patens* · *Ceratodon purpureus* · Adenine phosphoribosyl transferase

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Introduction

Gene targeting (GT) is a powerful approach in order to improve crop qualities and understand gene functions, two main goals in plant research.

The efficiency of gene targeting in an organism is defined as the ratio of targeted to random integration events observed upon transgenesis and reflects its capacity to integrate exogenous homologous DNA sequences at targeted genomic loci. In higher plants, GT efficiency hardly reaches 10^{-4} in a wild type background, which prevents the general use of such approach for precise genome modifications (Hanin and Paszkowski 2003).

An exception in the plant kingdom is the moss, *Physcomitrella patens*, in which integrative transformants resulting from homologous recombination are obtained almost at the same rate as that observed for *Saccharomyces cerevisiae* (Schaefer and Zrÿd 1997). Thus, gene knockout and allele replacement approaches are feasible in *P. patens* and places this moss in a unique position among model systems in multicellular eukaryotes (Schaefer 2002; Quatrano et al. 2007).

The moss, *Ceratodon purpureus*, is used as another moss model system to study gravitropic, phototropic and photomorphogenetic processes (Cove et al. 1997). Both mosses are in the same class of Bryopsida, suborder Funariineae for *P. patens* and suborder Dicranineae for *C. purpureus* (Vitt 2004).

In this study, we tested whether the high efficiency of gene targeting observed in *P. patens* is unique to this species, or if it can be achieved in other mosses. We thus compared the rate of gene targeting in *C. purpureus* and *P. patens* using genomic-based replacement vectors designed to target and disrupt the *APT* genes of both species. APT is an enzyme of the purine salvage pathway that converts adenine into AMP and its loss of function generates plants resistant to adenine analogues (Gaillard et al. 1998).

Here we show that the efficiency of GT in *C. purpureus* is at least 10 fold lower than in *P. patens*, but remarkably still 2 orders of magnitude higher than that observed in vascular plants. This situation is reminiscent of that observed in yeast and filamentous fungi and supports the hypothesis that high GT efficiencies could be a general feature of Bryophyte transgenesis.

Materials and methods

Plant material and culture conditions

Physcomitrella patens of the Gransden wild-type strain (Ashton and Cove 1977) and *C. purpureus* of the wild-type wt4 strain (Hartmann et al. 1983) tissues were propagated on PPNH4 medium, which corresponds to the minimal medium described by (Asthon et al. 1979) supplemented with 2.7 mM NH_4 tartrate. Cultures were grown in 9 cm Petri dishes solidified with 0.7% agar with a light regime of 16 h light/8 h darkness and a quantum irradiance of 80 μE m^{-2} s^{-1}.

Molecular cloning

Based upon the nucleotide sequence of the putative *APT* transcript [contig8338, id P004385, NIBB, PHYSCObase, http://moss.nibb.ac.jp/, (Nishiyama et al. 2003)], oligonucleotides PpAPTa (5′-CAGGA AGTGAAGATGTCG-3′) and PpAPTb (5′-TACTCG CCCTCATATTCCACC-3′) flanking the putative ATG start and TAA stop codons were designed. Using genomic *P. patens* DNA (Edwards et al. 1991) as starting template, a 1611 bp PCR fragment was amplified, and cloned into the TA-cloning vector pCR®II (Invitrogen) to produce pPpAPT. The Codehop software (Rose et al. 2003) was used to design degenerate PCR oligonucleotides based on APT-related plant sequences. Using *C. purpureus* genomic DNA as template, these oligonucleotides amplified a 1491 bp PCR fragment carrying the *CpAPT* gene, which was cloned into the TA-cloning vector pCR®II to produce pCpAPT. 5′ and 3′ RACE PCR were performed by TAIL-PCR according to Liu et al. (Liu et al. 1995) with nested specific primers CpAPTa (5′-CCAAAAAGTGGAGGCTATGG-3′) and CpAPTb (5′-GGAATGGCACGAATGCTGTCC-3′) for the 5′ RACE and nested specific primers CpAPTc (5′-CACTGGACTTAAAGTAGGTGG-3′) and CpAPTd (5′-TGAGGTTCACATCCTGATGTC-3′) for the 3′ RACE. For the construction of PpAPT-KO, fragment *Xba*I from pPpAPT (Fig. 1) has been deleted and replaced by the 35S-aphIV-CaMVter cassette from pCAMBIA1200 (http://www.cambia.org). For the construction of CpAPT-KO, fragment MfeI from pCpAPT (Fig. 1) has been deleted and replaced by the 35S-neo-CaMVter cassette from pHP 23b (Paszkowski et al. 1988). For both APT-KO constructs the antibiotic resistance cassettes are in the same orientation in regard to the transcription unit of the *APT* genes.

Protoplasts isolation

Protoplasts were isolated from 6-days-old protonemal cultures by incubation for 40 min in 1% Driselase (Fluka 44585) dissolved in 0.48 M mannitol. The suspension was filtered successively through 80 μm and 40 μm stainless-steel sieves. Protoplasts were sedimented by low-speed centrifugation (600 × *g* for 5 min at 20°C) and washed twice in 0.48 M mannitol. Protoplasts were then resuspended at 1.2×10^{-6} protoplasts/ml in MMM solution (8.8% mannitol, 15 mM $MgCl_2$, and 0.1% MES pH 5.6).

Transformation of protoplasts

Plasmid DNA was extracted with the Nucleobond XA kit (Macherey-Nagel, France). DNA from PpAPT-KO or CpAPT-KO were digested with *Eco*RI + *Bsr*GI and *Bst*EII + *Bsu*36I, respectively (Fig. 1), and further purified by phenol:chloroform extraction and ethanol precipitation. Moss protoplasts (300 μl per transformation for a total of 1.4×10^6) were transformed with 10 μg linearised DNA as previously described (Schaefer et al. 1997). Protoplasts were regenerated on

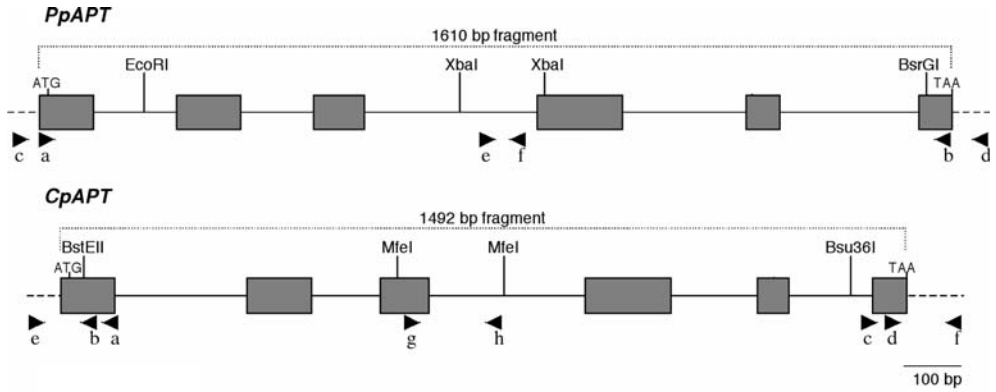

Fig. 1 Structure of the *PpAPT* and *CpAPT* Genes. Schematic representation of the genomic fragments containing the *APT* genes. Boxes are exons and lines are introns. Restriction sites used for the cloning of PpAPT-KO and CpAPT-KO cassettes and for the release of the gene-targeting fragment are indicated. Arrows indicate primers used in this study

$PPNH_4$ medium containing 0.33 M mannitol for a week and primary transformants were subsequently selected on $PPNH_4$ supplemented with 50 mg paramomycin/l (Duchefa) or 50 mg hygromycin B/l (Duchefa). Small pieces of protonemal tissue from primary *P. patens* and *C. purpureus* resistant clones were then transferred onto $PPNH_4$ non-selective medium for 15 days to differentiate replicative from integrative transformants. A second growth period of 2 weeks on selective medium (G-418 50 mg/l or HygB 100 mg/l) was then performed to unambiguously identify integrative transformants (Schaefer and Zrÿd 1997). Additional rounds of growth on selective and non-selective medium were required to identify *C. purpureus* integrative transformants (see results section).

Selection of 2FA resistant colonies

For selection of 2FA resistant colonies, small pieces of fresh protonemal tissue (approximately 2 mm diam) from stable transformants were transferred onto $PPNH_4$ medium supplemented with 2FA at 10 µM (for *P. patens*) or 50 µM (for *C. purpureus*) (gift of Dr Laloue, INRA Versailles, France). *P. patens* stable 2FA resistant colonies were selected directly whereas *C. purpureus* colonies had to be transferred three times on successive G418/2FA medium to be considered stable.

PCR analysis of the transformants

For PCR analysis, genomic DNA was extracted using a genomic DNA quick preparation procedure (Edwards et al. 1991). 50 mg of fresh tissue were ground in 400 µl extraction buffer (200 mM Tris/HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), and debris were removed by centrifugation (10,000 *g* for 1 min). DNA was precipitated by adding 300 µl 2-isopropanol to the supernatant (2 min at room temperature) followed by centrifugation at 10,000 *g* for 5 min. The pellet was resuspended in 100 µl TE buffer, and 1 µl of extract was used as template for PCR reaction in a final volume of 20 µl.

For both mosses, four specific primers were designed to check the presence of a complete APT gene. Two of them were located outside of the genomic fragment present on the KO vectors (PpAPTc, 5′-TTTTTGCGCTCGCTGTTTCTG-3′; PpAPTd, 5′- TAAATAATTCTGACCCAAAGT-3′ for *P. patens*, CpAPTe, 5′-ATCTGAGGTGGTCT TACGATTG-3′; CpAPTf, 5′-TCTGAGG GGCTTGAGATTGGC-3′ for *C. purpureus*), and the 2 others within the deleted *Xba*I (PpAPTe, 5′-TGTGATATCGATTGCTTTGTATG-3′ and PpAPTf, 5′-CGTCAGCACAAAATT GTCAAG GAT-3′ for *P. patens*) or MfeI fragments (CpAPTg, 5′-AATTGCACTTGCCATCGGTG-3′ and CpAPTh, 5′-GCACGGATTGAGTATTGACCA-3′ for *C. purpureus*, see Fig. 1). For the left and right border analysis of the insertions we used primers specific for the resistance cassettes (CaMVpro: 5′-GTGTCGTGCTCCACCATGT-3′, and CaMVter: 5′-TCTAATTCCTAAAACCAAAATCC-3′) and the primers specific for the *APT* sequences outside the transformation constructs (PpAPTc, PpAPTd, for *P. patens*, CpAPTe, CpAPTf, for *C. purpureus*).

The actin gene from *P. patens* was used as control using oligonucleotides act3U1 (5′-CGGAGAG GAAGTACAGTGTGTGGA-3′) and act3D1 (5′-AC CAGCCGTTAGAATTGAGCCCAG-3′) (Hara et al. 2001). For *C. purpureus* oligonucleotides CpACT1 (5′-ACGATGTTCCCGG GCATCGC-3′) and CpACT2 (5′-CTAGAAACACTTCCTGTGCAC-3′) were designed on an actin EST (AW098756) and used as control.

Results

Isolation of the *APT* genes of *C. purpureus* and *P. patens*

Based upon the sequence of ESTs coding for the APT of *P. patens*, a set of oligonucleotides flanking the putative ATG start and TAA stop codons were designed. Using genomic *P. patens* DNA as starting template, a 1611 bp PCR fragment was amplified, cloned and sequenced (Genbank no. DQ117987). In order to isolate the *APT* gene of *C. purpureus* we did a comparison of conserved amino acid sequences in known APT related proteins from various species including the APT from *P. patens* and designed two sets of degenerate oligonucleotides. Using genomic *C. purpureus* DNA as starting template, a 1491 bp PCR fragment was amplified, cloned and sequenced. 5′ and 3′ RACE PCR permitted the characterisation of a 1995 bp fragment (Genbank no. DQ265803). The *APT* genes from *P. patens* and *C. purpureus* were named respectively PpAPT and CpAPT, their introns/exons structure is shown on Fig. 1. Southern blot analysis revealed that the two genes are unique (result not shown). The *PpAPT* and *CpAPT* cDNAs show 89% identity and encode putative proteins of 184 amino acids. The deduced sequences of the PpAPT and CpAPT proteins showed 93% identity. The two proteins contained the regions involved in substrate binding as defined in (de Boer and Glickman 1991).

Stable versus unstable transformants in *P. patens* and *C. purpureus*

In order to estimate and compare gene targeting efficiency at the *PpAPT* and *CpAPT* locus, we constructed two disruption cassettes with the highest similarity in term of conformation as possible (see Materials and methods and Fig. 1). Concerning the PpAPT-KO vector, we deleted an internal *Xba*I fragment of 149 bp and replaced it by a hygromycin resistance cassette. For the construction of the CpAPT-KO vector, an internal Mfe1 fragment of 188 bp was deleted and replaced by a kanamycin resistance cassette. After digestion of the PpAPT-KO vector with *Eco*RI, which cleaves in intron I 3′ to the start codon, and *Bsr*GI, which cleaves in exon VI 5′ to the stop codon, the *PpAPT* disruption cassette shares 557 bp of identity with the *PpAPT* gene in the 5′ region and 674 bp in the 3′ region. After digestion of the CpAPT-KO vector with BstEII, which cleaves in exon I 3′ to the start codon and Bsu36I, which cleaves in intron V 5′ to the stop codon, the *CpAPT* disruption cassette shares 564 pb of identity with the *CpAPT* gene in the 5′ region and 631 pb in the 3′ region. Protoplasts of *P. patens* and *C. purpureus* were transformed with the digested PpAPT-KO and CpAPT-KO vectors (see Experimental procedures) and results are shown in Table 1. Primary

Table 1 Comparison of stable transformation and gene targeting efficiencies

Vector	*P. patens*				*C. purpureus*			
	RTF[a]/1000 colonies	Antib-R[b]	2FA-R[c]	GT[d] %	RTF[a]/1000 colonies	Antib-R[b]	2FA-R[c]	GT[d] %
PpAPT-KO	0.35 ± 0.15[e]	112	22	21.3 ± 2.0[e]	0.02 ± 0.015[e]	7	0	0
CpAPT-KO	0.07 ± 0.02[e]	21	0	0	0.58 ± 0.05[e]	187	2	1.05 ± 0.5[e]

[a] Relative transformation frequencies (RTF) express the frequency of antibiotic resistant transgenic strains in the whole regenerated population

[b] Antib-R[b]: number of colonies resistant to hygromycinB or G418

[c] 2FA-R[b]: number of colonies resistant to 2-fluoroadenine

[d] GT efficiencies express the frequency of 2-FA resistant among the whole population of antibiotic resistant strains

[e] Standard deviation was determined from 4 independent experiments

transformants resistant to hygromycin or paramomycin were transferred on basic medium for 15 days and then transferred back on antibiotics in order to discriminate integrative from replicative transformants (Ashton et al. 2000).

As expected, 84.3% of the primary resistant colonies were replicative transformants in *P. patens*. Similarly, in *C. purpureus*, 87.9% of the primary transformants were unstable, i.e., sensitive to the second round of selection. Noticeably, the identification of true integrative transformants in *C. purpureus* required several cycles of non-selective to selective growth. We can assume from those results that *C. purpureus* is probably able to replicate bacterial plasmids in the same way as *P. patens*, as previously proposed (Brücker et al. 2005). Furthermore, our data suggest that the mitotic segregation index of the episomal element is higher in *C. purpureus* than in *P. patens*. Integrative relative transformation frequencies (RTF: transformed colonies/regenerating colonies) observed in *P. patens* are consistent with those previously reported (Schaefer 2001), ranging between 10^{-4} and 10^{-5} with linearised non-homologous vectors (i.e., CpAPT-KO) and between 10^{-3} and 10^{-4} with linearised homologous vectors (i.e., PpAPT-KO). Integrative transformation rates observed in *C. purpureus* were comparable to those monitored in *P. patens* with the same difference between homologous and non-homologous transforming vectors (Table 1).

Phenotypic screen for *Ppapt* and *Cpapt* strains

In *A. thaliana*, loss of APT function generates plants resistant to the adenine analogues 2,6-diaminopurine (DAP) and 2-fluoroadenine (2FA) (Gaillard et al. 1998). Previous experiments have shown that loss of the APT function generates transgenic strains that are resistant to 250 μM DAP (Houba-Herin et al. 1997). Preliminary experiments with wild type strains grown on a range of 2FA concentrations (1 to 100 μM) established optimal selective concentration to correspond to 10 and 50 μM 2FA for *P. patens* and *C. purpureus* respectively (Fig. 2). We also evaluated the frequency of spontaneous resistance to 2FA in both species and found that it was lower than 10^{-6} protoplast-derived colony (result not shown). In order to estimate the rate of gene targeting to the *APT* locus, stable hygromycin and G418 resistant transformants were transferred on 2FA selective medium (Table 1). Phenotypes of wild type and *Ppapt* and *Cpapt* mutants on 2FA are shown on Fig. 2. The identification of 20% and 1% 2FA resistant colonies for *P. patens* and *C. purpureus* respectively within antibiotic resistant transformants strongly supports successful disruption of the *APT* gene. Based on this phenotypic screen, GT efficiencies were estimated to be around 20% in *P. patens* and 1% in *C. purpureus* (Table 1).

Molecular analysis of *apt* strains

To confirm that stable *P. patens* and *C. purpureus* 2FA resistant colonies were true *apt* mutants due to gene targeting, PCR analysis of representative clones has been done (Fig. 3). Using oligonucleotides present in the *APT* deleted fragment of PpAPT-KO or CpAPT-KO and oligonucleotides external to the genomic APT sequences present in the KO vectors (see Materials and methods procedures), PCR

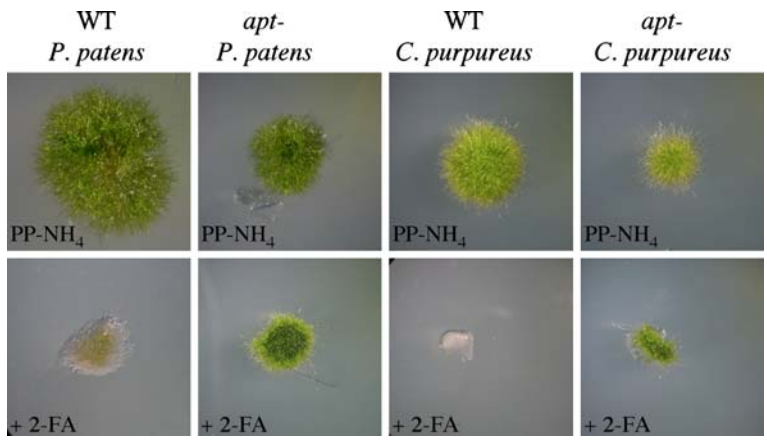

Fig. 2 Sensitivity to 2-fluoroadenine in *P. patens* and *C. purpureus*. Protonemal inocula of wild-type and *apt* mutants of *P. patens* and *C. purpureus* were transferred on $PPNH_4$ medium with or without 2FA (10 μM for *P. patens* and 50 μM for *C. purpureus*) and grown for 8 days

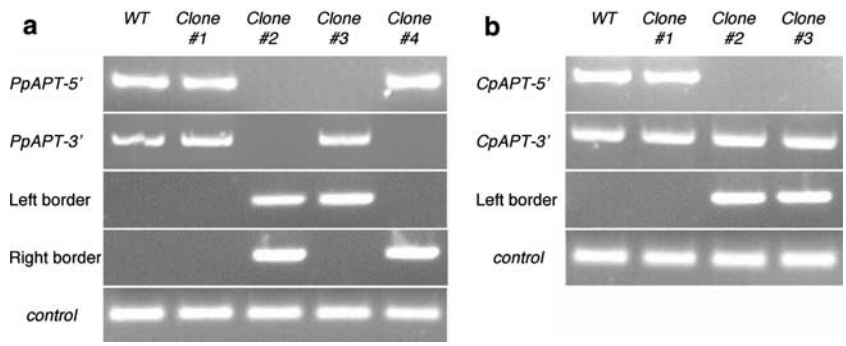

Fig. 3 Molecular analysis of the *APT* status in the transformants. (**a**) PCR analysis of transgenic strains. Clone no. 1 is Hyg^R and $2FA^S$, clone nos. 2 to 4 are Hyg^R and $2FA^R$. Fragments PpAPT5′ and PpAPT3′ were amplified using primers PpAPTc + PpAPTf and PpAPTd + PpAPTe, respectively. Left and right borders were amplified with primers flanking recombination sites and primers on the resistance gene (PpAPTc + CaMVpro, for left border and PpAPTd + CaMVter for right border). (**b**) PCR analysis of *C. purpureus* transgenic strains. Clone no. 1 is Hyg^R and $2FA^S$, clone no. 2 is Hyg^R and $2FA^R$. Fragments CpAPT5′ and CpAPT3′ were amplified using primers CpAPTe + CpAPTh and CpAPTf + CpAPTg, respectively. Left border was amplified with a primer flanking the 5′ recombination site and a primer on the resistance gene (CpAPTe + CaMVpro). Control PCR corresponds to the amplification of the actin gene from the same extracts

reactions were performed in order to check the integrity of the *APT* locus in the different colonies. PCR analysis of 30 stable hygromycin resistant and 2FA sensitive *P. patens* transformants and 30 stable G418 resistant and 2FA sensitive *C. purpureus* transformants were analyzed. Figure 3a (clone no. 1) and 3b (clone no. 1) shows an example of the PCR amplification obtained for the two series of colonies.

This analysis shows that amplification in these clones is similar to the one found in the corresponding wild-type colonies (Fig. 3a and b lane 1) and thus that the *APT* gene in these clones has not been targeted. The same type of PCR analysis was done on 15 stable hygromycin and 2FA resistant *P. patens* transformants and the two stable G418 and 2FA resistant *C. purpureus* transformants. The absence of one or of both of the PCR products and the presence of a left and/or right recombined borders were taken as evidences for the targeted integration of the vector at the corresponding APT locus by insertion or replacement, respectively. Our results demonstrate that the *APT* gene has been targeted in all the clones tested, with 7 *P. patens* clones showing a gene replacement (Fig. 3a clone no. 2) 3 clones showing a 5′ integration (clone no. 3) and 5 clones showing a 3′ integration (clone no. 4). Similarly, the two 2FA stable resistant clones for *C. purpureus* showed a 5′ integration (Fig. 3b clone nos. 2 and 3). We did not detect GT events when we transformed *P. patens* protoplasts with the *C. purpureus* cassette or reciprocally, despite the fact that the coding sequence of the *APT* genes shared 89% identity. We recently showed that 3% divergence in targeting sequences lead to a 22-fold reduction of GT efficiencies in wild type *P. patens*, and that this reduction was associated with an active mismatch repair (MMR) system, since GT efficiencies with divergent sequences were not reduced in *P. patens msh2* mutants (Trouiller et al. 2006). The relation between the MMR system and GT efficiencies is well established in budding yeast and seems similar in *P. patens*. The inability to target *C. purpureus* genes with *P. patens* targeting sequences and reciprocally was anticipated and most likely reflects the role of the MMR system in targeted transgenesis in moss.

Discussion

In this study, phenotypic and molecular analyses provided reliable data that enabled us to assess gene targeting efficiency in both moss species. Our results demonstrate that gene disruption by GT is possible in wild type *C. purpureus* at an efficiency approximately two orders of magnitude higher than that obtained to date in higher plants (10^{-2} vs. 10^{-4}). Yet, GT efficiency in *C. purpureus* is ca. one order of magnitude lower than that achieved in *P. patens*

(1.05% and 20.8%, respectively, see Table 1). This situation is reminiscent of the different targeting efficiencies observed in distinct yeast species or filamentous fungi (Schaefer 2001). We therefore conclude that efficient GT (i.e., above 10^{-2}) is a property of transgenesis shared by at least 2 different moss species and could reflect a general feature of Bryophyte transformation.

GT efficiencies determined in wild type vascular plants hardly reaches 10^{-4}, and this constitutes the major methodological limitation to the application of targeted mutagenesis in plant functional genomics (Puchta 2003). Over the last ten years, improvement of GT efficiencies has been attempted using both loss of function (null allele) or gain of function (overexpressors) mutants with limited success (Puchta 2003). Recently however, Shaked and co-workers have shown that overexpression of the chromatin remodeling protein Rad54 from *S. cerevisiae* in *A. thaliana* increased GT frequencies in average 28 fold (Shaked et al. 2005). Yet this promising set of data needs to be confirmed on other independent loci, in other plant species and following direct gene transfer of naked DNA into plant cells to establish a general method to routinely achieve GT in plants. The authors suggest that chromatin remodeling could be the rate-limiting step of this process. If this assumption is true, it would imply that the two mosses studied here may have unusual chromatin dynamics. In this context, a comparison between *C. purpureus* and *P. patens* with higher plants in term of homologous recombination machinery should provide tools to develop novel approaches to achieve good frequencies of gene targeting in plants and thus the construction of ''safer'' transgenic crops.

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