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REVIEW

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A tool for understanding homologous recombination in plants

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Abstract Attempts for establishing an efficient gene targeting (GT) system in seed plants have hitherto not been successful. In contrast, GT based on homologous recombination is highly efficient in *Physcomitrella*, making this moss a novel tool in reverse genetics. However, why homologous and illegitimate recombination are differently regulated between *Physcomitrella* and seed plants is still enigmatic. Here we update the state of the art of GT in *Physcomitrella* and discuss approaches to unravel this enigma. Identification of molecular factors significantly enhancing GT and their subsequent transfer to crop plants will have a great impact on plant biotechnology by enabling precise genetic engineering. *Physcomitrella* appears to be the most useful model system in this context.

Keywords *Arabidopsis thaliana* · Cell cycle · Gene targeting · Moss · *Physcomitrella patens*

Introduction

DNA recombination processes are responsible for interchromosomal exchange of genetic information during meiosis, for repair of DNA double strand breaks (DSBs) as well as for integration of foreign DNA. Whereas the first is confined to meiotic cells, both DSB repair and foreign DNA integration occur in somatic cells and are believed to be controlled by the same recombination machinery (Mengiste and Paszkowski 1999; Vergunst and Hooykaas 1999). The efficient repair of DNA DSBs is essential for cell survival. It is achieved either by mechanisms resulting in homologous recombination (HR), which requires a homologous template of the broken DNA and leads to precise repair, or by processes using non-homologous templates or just ligating any two double-stranded DNA ends, resulting in illegitimate recombination (IR). The enzymes involved in these processes are evolutionarily conserved; however, their relative activity varies considerably between different organisms. Whereas HR is the dominating process in bacteria and unicellular eucaryotes with yeast as an outstanding example, DSBs in multicellular eucaryotes are predominantly repaired via IR (Mengiste and Paszkowski 1999; Vergunst and Hooykaas 1999). This has a great impact on the genetic engineering of crop plants, since the prevalent mechanism for DSB repair is also used for the integration of foreign DNA. Here, IR is leading to random integration of the transgene, often in unpredictable copy numbers (Vergunst and Hooykaas 1999; Kumar and Fladung 2001), whereas HR results in the precise integration of transgenic DNA at the homologous site of the host genome, thus allowing gene targeting (GT). GT, on the other hand, is widely used as a basic tool in functional genomics for unravelling unknown gene functions via reverse genetics.

Great efforts have been undertaken for establishing a GT system in plants (Puchta 2002) but, to date, all attempts have been unsuccessful, mainly due to the fact that in flowering plants IR is the prevailing process for transgene integration. However, one organism known within the plant kingdom, the moss *Physcomitrella patens*, exhibits extraordinarily high GT rates, comparable to those known for yeast (Schaefer 2001). Thus, *Physcomitrella* has also been termed "the green yeast" and consequently emerged as a standard tool for analysing gene functions via reverse genetics in plants (Reski 1998a). Moreover, this is a tempting situation for research on molecular factors regulating HR in plants, as only the high GT rate of this moss provides a powerful model system in the search for molecular factors promoting this phenomenon. However, in contrast to the rising number of investigations in which GT in *Physcomitrella* has been used as a tool, to date only very little data have been

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published analysing the unique recombination machinery of *Physcomitrella* at the physiological or the molecular level.

In this review we discuss the state-of-the-art of gene targeting in *Physcomitrella* and suggest approaches for future research on identification of molecular factors regulating the relative activities of HR and IR in plants.

For details on other focal points within the field of HR and GT in plants, we recommend several reviews published during the last years: Mengiste and Paszkowski (1999) and Vergunst and Hooykaas (1999) provide comprehensive summaries on various aspects of recombination in the plant genome; Gorbunova and Levy (1999) and Ray and Langer (2002) describe in detail the different models of the molecular mechanisms of recombination; approaches for improvement of GT in flowering plants are summarized by Kumar and Fladung (2001) and Puchta (2002); Schaefer (2001, 2002) gives an overview of GT in *Physcomitrella*.

Physcomitrella as a model system

All major biological processes in *Physcomitrella* are similar to those in flowering plants despite 450 million years of divergent evolution (Theissen et al. 2001). However, due to the former's relatively simple body plan and well-defined physiological reactions, many biological questions can be answered in a more straightforward manner using *Physcomitrella* rather than with flowering plants as the experimental system. A major characteristic of moss development is the predominance of the gametophytic haploid phase. After germination of the haploid spore, the two-dimensionally growing filamentous protonema develops, consisting of two cell types—chloronema and caulonema. The emergence of buds marks the transition to the three-dimensionally growing gametophore, which later on produces the sex organs—archegonia and antheridia. Following fertilization of the egg cell with a spermatozoid, the tiny diploid sporophyte develops, which bursts in the mature stage to release the haploid spores.

In addition to this comparatively simple plant architecture, very pronounced and well-defined physiological and developmental reactions of *Physcomitrella* to environmental triggers—for example, growth regulators, light and temperature—have been described. Thus, *Physcomitrella* has been used to study questions from various fields of classical plant physiology. These have been discussed in several reviews on this topic (Bopp and Bhatla 1990; Cove and Knight 1993; Cove et al. 1997; Reski 1998b). More recent publications in this field comprise polarity induction (Bhatla et al. 2002), protoplast regeneration (Schween et al. 2003a) and photoperiodism (Hohe et al. 2002).

During the last decade, the molecular genetics of *Physcomitrella* has been developed, which has been reviewed in detail by Reski (1999) and Schaefer (2001, 2002). Recently, large-scale projects on functional genomics of *Physcomitrella* were set up. Nishiyama et al. (2000) were the first to report on the production of a tagged mutant collection of *Physcomitrella* for the identification and isolation of genes. By transforming the wild-type *Physcomitrella* with *Physcomitrella* genomic DNA tagged by a minitransposon the authors produced 5,264 randomly tagged mutants with 3.8% showing an altered phenotype. Likewise, 5,757 gene-trap lines were generated using the *uidA* reporter gene, of which 2.7% showed β-glucuronidase (GUS) activity in some tissue. Hiwatashi et al. (2001) improved the gene-trap system and also developed an enhancer trap system again using the *uidA* reporter gene. Here, 4.1% of 5,637 gene-trap lines and 28.8% of 3,726 enhancer-trap lines showed GUS activity. These authors also demonstrated the successful identification of a trapped gene by RACE-polymerase chain reaction (PCR). Egener et al. (2002) reported the generation of a tagged *Physcomitrella* mutant collection using transformation of the wild type with mutagenized moss cDNA libraries, thus aiming to produce a collection of *Physcomitrella* transformants with insertion mutations in most of the expressed genes. Here, 16.2% of the first 16,203 transformants clearly differed from the wild type, with both metabolic mutants (Fig. 1) and morphologically differing plants (Fig. 2) occurring. The high rate of phenotypically noticeable mutants in this approach compared to that obtained by

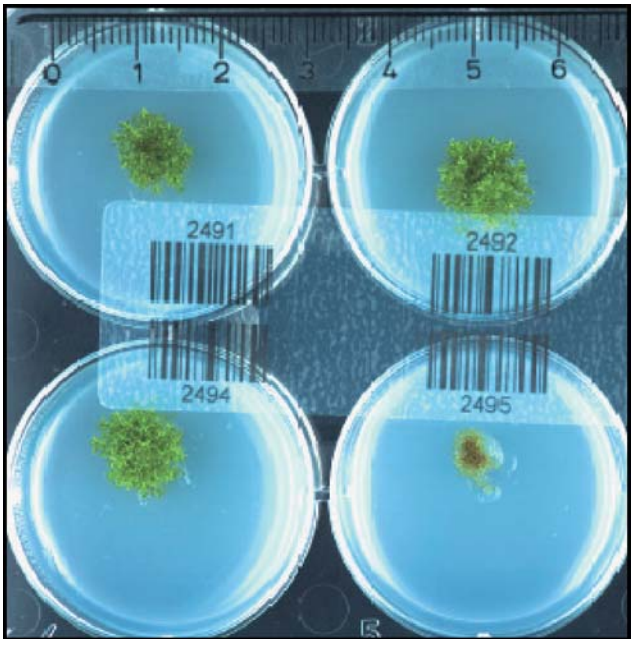

Fig. 1 Metabolic mutants of *Physcomitrella* obtained by transfection with a gene disruption library. *Physcomitrella* plants derived from transfection with a gene disruption library were split in two parts, which were then regenerated on minimal Knop medium with and without supplements. The picture shows four independent transformants cultured on minimal medium, one of which (*bottom right*) clearly displays retarded growth. All four plants grew equally well on supplemented medium (data not shown). The *scale bar* on *top* indicates size in centimetres. (Figure taken from Egener et al. 2002)

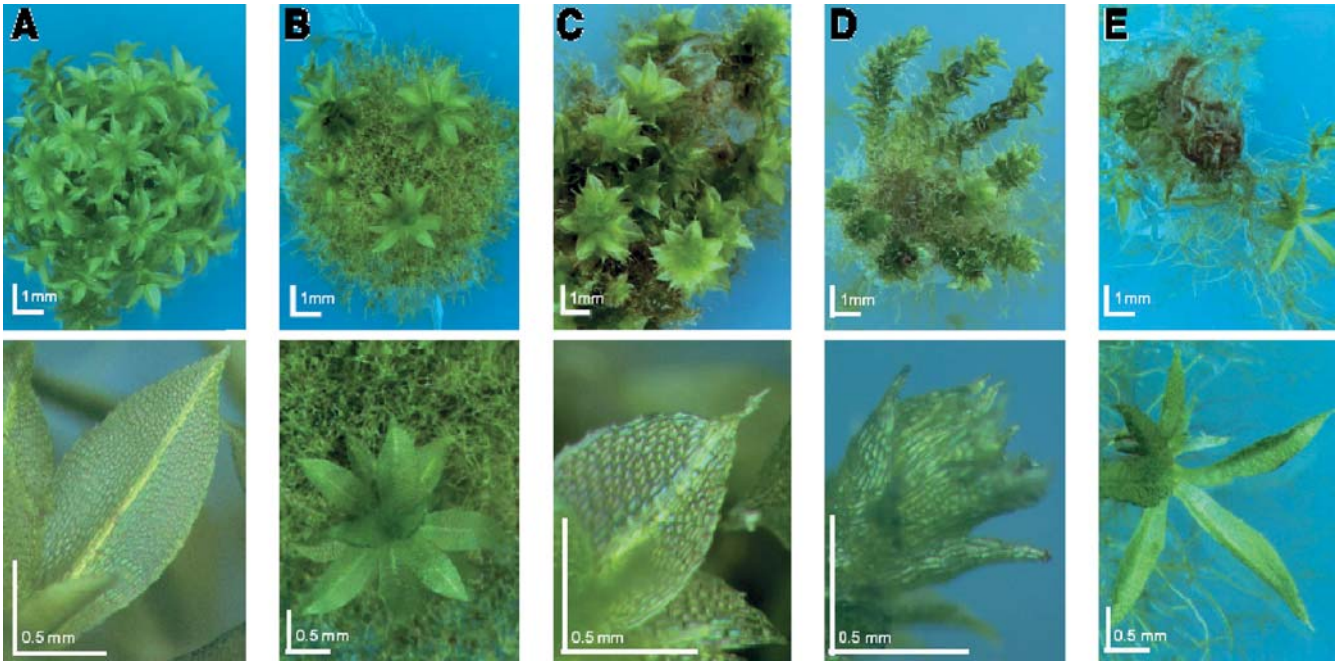

Fig. 2A–E Deviating phenotypes of *Physcomitrella* induced by gene-disruption library transformation. Wild-type and transformed *Physcomitrella* plants were grown on minimal Knop medium to induce differentiation and development of gametophores. For each plant an overview (*upper row*) and a close-up (*bottom row*) is shown. **A** Haploid wild-type moss completely covered with leafy gametophores and close-up of wild-type leaf. **B** Transformant BC22189 affected in differentiation, mostly comprising a filamentous protonema with reduced number of gametophores, but normal leaf morphology (haploid). **C** Transformant BC11280 showing retarded growth, a reduced number of gametophores and altered leaf morphology (twisted tips of leaves, haploid). **D** Transformant BC1015 displaying an altered growth habit reminiscent of the waterweed *Elodea*. **E** Transformant BC22288 showing retarded growth and elongated, narrow leaves (polyploid). (Figure taken from Egener et al. 2002)

Nishiyama et al. (2000) is probably due to the use of mutagenized cDNA instead of genomic DNA, thus raising the probability of targeting expressed genes.

In conjunction with these large-scale genomics approaches, several expressed sequence tag (EST) sequencing programs were set up. In a joint program of the University of Leeds (UK) and Washington University in St. Louis (USA), a database of 30,000 ESTs was generated, while the laboratory of Hasebe (Okazaki, Japan) has released 50,000 ESTs into public databases to date. Both collections are publicly available. In a collaboration between Freiburg University (Germany) and BASF Plant Science, a proprietary EST library from different developmental stages of *Physcomitrella* was produced comprising 110,000 clones, that were assembled to 33,000 distinct EST clusters. A detailed bioinformatic analysis of this library by Rensing et al. (2002) revealed that the *Physcomitrella* transcriptome contains about 25,000 genes, which is within the range of the *Arabidopsis* gene content of about 26,000 genes (The *Arabidopsis* Genome Initiative 2000). However, despite the similar number of genes, *Arabidopsis* homologues have been identified for only 50% of the *Physcomitrella* genes so far; 11,600 *Physcomitrella* EST clusters do not match significantly to any known sequences, thereby showing that *Physcomitrella* is a rich source of unknown plant genes. It has been shown that *Physcomitrella* possesses gene families similar to those found in flowering plants, however the average gene family is only half as large in comparison to *Arabidopsis*, which means that the moss possesses a high number of unique genes. The genome size of *Physcomitrella* has been estimated to be 511 Mbp (Schween et al. 2003b), which is about four times the size of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative 2000) in terms of haploid genome sets and within the same range as the rice genome (Goff et al. 2002; Yu et al. 2002). Thus, the *Physcomitrella* genome is less tightly packed than the *Arabidopsis* genome. However, its predominant haploid state and high number of unique genes make *Physcomitrella* an attractive model organism for functional genomics approaches, since the probability that gene targeting approaches will result in detectable phenotypes is very high. Moreover, as outlined in detail below, this moss is so far the only known organism within the plant kingdom that enables gene targeting based on homologous recombination.

Both the characteristics of *Physcomitrella* as a model organism for studies on plant development as well as physiology and its unique potential in molecular approaches have contributed to its emergence as a model with a high potential in several fields of plant biology.

Homologous recombination in plants

GT is achieved through the precise replacement of a gene by (partially) homologous foreign DNA via HR. Since the processes involved in the integration of foreign DNA are basically identical with those responsible for DSB repair, a thorough understanding of the recombination processes following DNA DSBs is the basis for improving the GT efficiency in plants. Several models of recombination processes have been set up to explain data obtained from experiments dealing with DSB repair, which will be presented here only briefly (for details see Puchta and Hohn 1996; Gorbunova and Levy 1999; Vergunst and Hooykaas 1999; Ray and Langer 2002).

The repair of DSBs following transposon excision, ionizing radiation or endonuclease activity, for example, is critical for cell survival, because these lesions have to be repaired to ensure complete replication of the genome. One possible mechanism is non-homologous end-joining (NHEJ)—i.e. the ligation of any two double-stranded DNA ends. Since this process does not require a homologous template, it is error-prone and leads to rearrangements of the genome. Single-strand annealing (SSA) involves the degradation of the double-stranded ends by exonucleases, leading to single-stranded ends, which are subsequently annealed at sites of micro-homologies resulting in a deletion of the region between the microrepeats. During synthesis-dependent strand annealing (SDSA) single-stranded 3′ ends invade a (homologous or non-homologous) template and prime DNA synthesis; the newly synthesized DNA is released from the template, resulting in long stretches of single-stranded DNA and a migrating replication bubble. When the newly synthesized DNA is displaced from the template, the repair is completed via an SSA-like mechanism. In the case of a non-homologous template, so-called filler DNA is generated, which can also be of a "patchwork-type" if template switches have occurred. If a homologous template was used, SDSA leads to homologous recombination via gene conversion. The classical DSB repair model (DSBR) involves the formation and subsequent resolution of Holliday junctions, resulting in conversion or crossover.

DNA repair mechanisms leading to IR are the most prominent DSB repair processes in flowering plants, whereas HR via DSBR is rare (Vergunst and Hooykaas 1999; Ray and Langer 2002). HR in somatic cells of plants has been investigated using experimental designs in which disrupted marker or selection genes had to be repaired via HR. Although the numbers on recombination frequencies from these publications can hardly be compared due to different experimental setups using different constructs with different marker genes, the estimated recombination frequencies were consistently low—ranging between 10^{-4} and 10^{-7} events per cell division for intrachromosomal recombination (reviewed by Puchta and Hohn 1996). Recombination frequencies were within the same range using other recombination substrates such as ectopic homologous sequences (Puchta 1999) or allelic homologous sequences (Gisler et al. 2002). Moreover, although abiotic and biotic stress did enhance somatic recombination frequency several fold (Lebel et al. 1993; Puchta et al. 1995; Lucht et al. 2002), attempts to use stress factors for enhancing GT efficiency have not yet been successful (Lebel 1994). Thus, the highest GT rates reported for flowering plants range from 0.01% to 0.1% amongst transgenics (Lee et al. 1990; Miao and Lam 1995; Kempin et al. 1997).

Most of the research on molecular mechanisms of recombination in eucaryotes has been performed in yeast. From these data, a model has been deduced that has recently been reviewed by Ray and Langer (2002) with respect to its validity for plants. According to this model, the DNA ends of the DSB serve as a substrate for the multiple-enzyme complex Rad50-Rad58(Mre11)-Rad60(Xrs2), which resects the ends by exonuclease activity to exposed single strands. Interaction with Rad52 leads to the assembly of another multiple enzyme complex, the Rad51-Rad54-Rad55-Rad57 complex, which finally leads to the homology-dependent repair processes of DSBR or SDSA. If Rad52 is not available, a Ku70-Ku80 complex binds to the DNA ends, which allows the recruitment of DNA ligase IV (Dn14) and its accessory factor, Lif1 (XRCC4). In this latter case, the DNA ends are joined via NHEJ. According to this model DSBR/SDSA and NHEJ compete for available DNA ends, which is mirrored at the molecular level by competition between Rad52 on the one hand and Ku70-Ku80 on the other. In flowering plants, homologues of the genes involved in these processes have been identified for Rad51 (Doutriaux et al. 1998; Stassen et al. 1997; Franklin et al. 1999), Dn14 and XRCC4 (West et al. 2000). However, to date, no Rad52 homologue has been identified in plants, and Ray and Langer (2002) discuss whether the absence of Rad52 might be correlated with low levels of HR in flowering plants.

Gene targeting in *Physcomitrella*

As outlined above, the highest GT rates reported for flowering plants range from 0.01% to 0.1% (Lee et al. 1990; Miao and Lam 1995; Kempin et al. 1997). In sharp contrast, GT rates in the moss *P. patens* have been reported to be between 4% and 95% amongst transgenics (Schaefer 2001) using PEG-mediated protoplast transformation with insertion or replacement vectors (for description of the transformation method see Schaefer et al. 1991, 1994; Hohe et al., submitted). Consequently, soon after the first report on efficient homologous recombination in *Physcomitrella* by Schaefer and Zryd (1997), *Physcomitrella* developed into a powerful tool in plant functional genomics for the analysis of gene functions by reverse genetics. In several quite diverse fields of plant biology, gene knockout mutants of *Physcomitrella* have been generated and their phenotype used to unravel or confirm gene functions. The first knockout mutant of *Physcomitrella* showing a clearly altered phenotype was

reported by Strepp et al. (1998). Targeting of the *fts*Z2-1 gene resulted in a mutant containing only one big macrochloroplast per cell, thus clearly identifying FtsZ2-1 as the first organelle division protein in any eucaryote. Other biological processes in which GT in *Physcomitrella* has been used to analyse gene functions include the biosynthesis of fatty acids (Girke et al. 1998; Zank et al. 2002), protein degradation (Girod et al. 1999), photomorphology (Imaizumi et al. 2002) and sulfate assimilation (Koprivova et al. 2002).

It has recently been demonstrated that even multiple gene knockouts can easily be generated in *Physcomitrella* by transformation with a mixture of the relevant knockout constructs (Hohe et al., submitted). In this study five different knockout constructs were mixed in equimolar amounts prior to transformation and the resulting plants analysed by PCR with respect to the knockout of two of the targeted genes. It was shown that the GT rate in these two single loci was in the same range as the rates obtained in parallel transformations in which only one of the knockout constructs was used, indicating that the different constructs did not interfere or compete during transformation and recombination. Moreover, 4.8% of the generated mutant plants showed double knockouts in both genes. Using this easy method of transformation with a mixture of the knockout constructs of interest, researchers can avoid the laborious step-by-step procedures involving several transformation steps using different selection markers. In vertebrate cells and yeast, multiple gene disruptions are used for the functional analysis of epistasis group genes (Morrison and Takeda 2000; Sung et al. 2000). Using *Physcomitrella*, this field is now also open for plant functional genomics.

The above-mentioned GT approaches of single genes in *Physcomitrella* have been reviewed in detail by Schaefer (2001, 2002), who also gives detailed comparisons of the different knockout constructs regarding vector construction and DNA topology and correlates these with GT efficiency. However, these data remain vague since the experimental setups were quite diverse in the different publications, and often the number of replications was too low for exact numbers to be calculated. Our data, obtained in a high-throughput approach at Freiburg University in collaboration with BASF Plant Science, give new insights into the dependence between GT efficiency and factors involved in the transformation procedure. In this approach, a total of 59 different genes were targeted using linearized gene disruption constructs based on cDNA clones. On average, a GT rate of 26% amongst transgenics was achieved. Knockout mutants were identified for 55 out of these 59 target genes (80%). No knockout mutants were obtained for only four of the candidate genes, which might be caused by a lethal effect of the disruption of these genes. The maximum GT rate observed for a single cDNA-based construct was 88%. A GT efficiency of less than 5% was obtained for only eight out of the 59 target genes.

In a parallel approach three selected knockout constructs were compared that differed in length and organization of the corresponding genomic loci (Hohe et al., submitted). A knockout construct with a length of 1.9 kb corresponding to an intronless genomic locus resulted in a GT rate of 45.6%. Another construct of identical length but with its genomic locus disrupted by introns yielded only 16.1% of knockout mutants. In contrast, a GT rate of 31.1% was achieved using a construct corresponding to an intronless genomic segment with a length of only 1 kb. These data demonstrate that both factors, DNA length and organization of the genomic locus, affect GT efficiency. However, the genomic locus organization seems to be the dominant factor or, in other words, the presence of long continuous stretches of the genomic sequence will lead to a high GT rate.

Why is GT so efficient in *Physcomitrella*?

Although GT in *Physcomitrella* has become a routine tool in plant functional genomics and the methods have been optimized as described above, to date it is mere speculation why GT is working so efficiently in *Physcomitrella* but not in flowering plants. According to the molecular analyses of the targeted loci, GT is clearly accomplished by HR events in *Physcomitrella*. Thus, the question arises as to why the recombination machinery is so efficient in *Physcomitrella* but not in flowering plants. Are there specific enzymes in the moss, or is the regulation of recombination enzymes unique?

A first hint on the special organization of the *Physcomitrella* recombination machinery is given in two recent publications (Ayora et al. 2002; Markmann-Mulisch et al. 2002): Two highly homologous *RAD51* genes have been found in *Physcomitrella*. The corresponding proteins are 94% identical in their sequence, but it remains to be investigated whether this gene duplication is restricted to *RAD51* or reflects a genome duplication. Even more surprising than the gene duplication is their intronless structure, which is in contrast to all other *RAD51* genes known thus far from multicellular eucaryotes. Since in budding yeast and fission yeast a meiosis-specific expression of the *RAD51* homologue *DMC1* has been correlated with the presence of introns, Markmann-Mulisch et al. (2002) assumed that the unique organization of the *Physcomitrella RAD51* homologues might be responsible for a recombination apparatus differing from that of all other multicellular eucaryotes.

As discussed by Ray and Langer (2002), it has been speculated that the absence of *RAD52* might be correlated with low levels of HR in flowering plants. Thus, the *Physcomitrella* EST databases should now be used to search for homologues of factors known to be involved in recombination in other organisms. Knockout of these candidate genes will result in mutant plants that can be analysed for alterations in their GT efficiency.

In *Arabidopsis*, the MIM protein belonging to the group of SMC (structural maintenance of chromosomes)-related proteins has been identified to affect HR frequencies (Mengiste et al. 1999). Although overexpression of

the *MIM* gene resulted only in a small increase in the HR frequency (Hanin et al. 2000), Vergunst and Hooykaas (1999) argue that the large differences in HR frequencies between extrachromosomal and intrachromosomal recombination may indicate the importance of the chromatin structure, possibly due to accessibility of the DNA for recombination enzymes. To our knowledge, to date nothing is known about the chromatin structure of *Physcomitrella* with respect to HR frequencies. A search for *Physcomitrella* homologues of relevant genes with subsequent knockout and analysis of the resulting mutant plants with respect to their GT efficiency would allow the importance of this factor to be assessed.

Puchta (2002) also assumed a connection between the unique ability of *Physcomitrella* to replicate extrachromosomal plasmid DNA (Ashton et al. 2000) and HR frequency: this might be a hint of a kind of "activation" of foreign DNA for homologous interactions after uptake by the *Physcomitrella* cell.

Schaefer and Zryd (1997) correlated the efficient homologous integration of transgenes with the haploid state of the moss protonema. However, in yeast, both haploid and diploid strains exhibit high HR rates (Mengiste and Paszkowski 1999). Moreover, in tobacco, no effect of the ploidy level on the rate of HR has been found (Lebel 1994; Risseeuw et al. 1997).

Also, the gametophytic state itself does not seem to increase GT rates, since during the common transformation method for *Arabidopsis*, *Agrobacterium* is applied to the bolting plant. Thus, the transforming DNA probably reaches the female gametophyte (Bechtold et al. 1998); however, this method did not enhance GT rates in *Arabidopsis* (Mengiste and Paszkowski 1999).

Reski (1998b) speculated on interactions between cell-cycle phases and HR frequencies in *Physcomitrella*. Schween et al. (2003b) showed that chloronema cells of *Physcomitrella* are specifically arrested at the G2/M boundary of the cell cycle. In contrast, caulonema cells are predominantly arrested at the G1/S transition, as is known for cells of flowering plants. Since protoplasts for transformation are obtained from chloronema-rich cultures (Schaefer et al. 1991; Hohe et al. 2001; Hohe and Reski 2002), most of the transformed cells are probably at the G2/M transition of the cell cycle. Indeed, it has been shown in vertebrate cells that the relative activity of HR and IR is cell-cycle phase-specific, with HR being more efficient during the S and G2 phases (Takata et al. 1998; Dronkert et al. 2000). However, it remains to be demonstrated that HR frequencies differ for chloronema and caulonema cells.

An interesting approach would also be to analyse GT frequencies in other mosses or bryophytes. This will also be interesting in the context of the evolutionary importance of recombination processes. In order to explain the low HR frequency of flowering plants, it has been argued that the more error-prone DSB repair mechanisms of flowering plants allow more rapid genomic changes, which is important for adaptation to changing environments, since plants cannot move or act via behavioural changes (Gorbunova and Levy 1999). On the other hand, it has been argued that high HR frequencies would cause severe problems of genomic integrity in plant genomes that contain dispersed repeated DNA (Ray and Langer 2002). The special regulatory mechanisms of recombination processes in plant cells might therefore have evolved to keep a subtle balance between genome plasticity and stability. However, none of these theories takes into consideration the special case of *Physcomitrella*. Might its uniquely high HR frequency have contributed to the evolutionary stability of this ancient plant—or, in contrast, did it enable flexible evolutionary adaptation to changing environments?

As discussed above, there are multiple approaches by which to analyse potential reasons for the differences in HR frequencies between flowering plants and *Physcomitrella*. Getting hold of any physiological or developmental factor that is modulating HR frequencies will be the basis for isolation of the relevant genes. Thus, apart from using *Physcomitrella* as a tool in plant reverse genetics by generating knockout plants, this moss should now be used as a tool for understanding HR in plants. Transfer of the unique characteristic of high HR frequencies to crop plants will have an outstanding biotechnological impact. By overcoming the current major drawbacks of transgenic plants—i.e. random integration of transgenes and unpredictable expression levels—by precise genetic engineering, security and public acceptance of transgenic crop plants will increase.

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