Rescue and characterization of episomally replicating DNA from the moss *Physcomitrella*

Eva Murén[1], Anders Nilsson[1], Mikael Ulfstedt, Monika Johansson[2], and Hans Ronne[3]

Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, SE-751 23 Uppsala, Sweden

Edited by John A. Carbon, University of California, Santa Barbara, CA, and approved September 25, 2009 (received for review July 17, 2009)

The moss *Physcomitrella* is unique among plants in that it permits efficient gene targeting by homologous recombination. Furthermore, transformed DNA can replicate episomally in *Physcomitrella*. Here we show that episomally replicating DNA can be rescued back into *Escherichia coli*, and we use such rescue to study the fate of the transformed DNA. Significantly, plasmids rescued from moss transformed with circular DNA are identical to the original plasmid, whereas plasmids rescued from moss transformed with linearized DNA frequently have deletions created by direct repeat recombination. These events are highly predictable in that they target the longest direct repeat on the plasmid if this repeat is at least 12 bp. Episomal transformants obtained with linearized DNA show a more than 1,000-fold amplification of the DNA whereas transformants obtained with circular DNA have much lower copy numbers. Most episomal transformants quickly lose the plasmid in the absence of selection, but a semistable type of transformant that loses the plasmid at a much lower frequency was also observed. The consistent rescue of the original plasmid, or of predictable derivatives thereof, suggests that molecular genetics methods which rely on shuttle plasmids are feasible in *Physcomitrella*.

episomal replication | plasmid rescue | recombination | shuttle plasmid

The discovery of gene targeting by homologous recombination (1) and the development of yeast shuttle plasmids (2–4) were key advances in eukaryotic molecular genetics. Gene targeting made reverse genetics possible, and shuttle plasmids made it easy to clone both yeast genes and genes from other eukaryotes by complementation of yeast mutants (5). Furthermore, the effects of overexpressing a gene could be studied by using plasmids that replicate in high copy number; by screening genomic libraries made in such plasmids, dosage suppressors of yeast mutants could be cloned, one of the most powerful methods available in molecular genetics (6).

The finding that gene targeting is feasible in the moss *Physcomitrella* (7) transformed this small plant into an important model organism (8–11). Interestingly, early studies also showed that foreign DNA can replicate episomally in *Physcomitrella* (12–15). Three types of transformants were described by Schaefer (14). Class I have the DNA integrated into the moss genome and further studies of these transformants led to the discovery of gene targeting in moss (7). Class II, which comprise most transformants, quickly lose the marker under nonselective growth. Class III transformants also lose the marker in the absence of selection, but the rate of loss is lower (14). Further studies by Ashton et al. (15) showed that foreign DNA can replicate episomally in moss for at least 10 years if selection is maintained. Southern blots and PCR experiments suggested that the episomal DNA forms concatemers with 3–40 copies of the plasmid (14, 15). A notable finding in both studies was that moss DNA does not seem to be required for replication, unlike the case in yeast. It was, however, suggested that class III concatemers might contain moss-derived sequences, which could stabilize replication (14).

A key feature of any shuttle plasmid system is the ability to rescue the plasmid back into *Escherichia coli* without rearrangements or insertions of host DNA. Here we show that episomally replicating plasmids in *Physcomitrella* can be rescued back into *E. coli* from undigested *Physcomitrella* DNA. Significantly, plasmids rescued from moss transformants obtained with circular DNA are identical to the original plasmid. We conclude from this that molecular genetics methods that rely on shuttle plasmids are feasible in *Physcomitrella*.

Results

We set out to study the fate of different plasmids after transformation into moss. Previous work has suggested that YAC vectors are more prone to stable episomal replication in *Physcomitrella*, possibly due to the yeast centromere (*CEN*) or telomere (*TEL*) elements on these plasmids (14). To assess the effect of either element, we used 3 related plasmids (Fig. 1). The first, pEM209, contains *CEN4*, *ARS1* (a yeast origin of replication) and 2 *TEL* elements from pYAC4 (16). pEM207 contains only *CEN4* and *ARS1*, and pEM203 contains none of these elements. All 3 plasmids share the bacterial origin of replication, the Amp^R selection marker, and the *npt II* cassette used for selection of G418 resistance in moss.

We also examined the effects of using either linearized or circular supercoiled DNA for transformation. DNA used for gene targeting in both yeast and moss is linearized before transformation since free DNA ends are recombinogenic (1, 7). Furthermore, it has been reported that the stability of YAC-based plasmids in moss is enhanced if they are linearized (14). For these reasons, and since we wanted to study how free DNA ends are processed in moss, each plasmid was transformed into moss either as supercoiled circular DNA or after linearization. To linearize pEM203 and pEM207, we used a unique BamHI site in the pUC19 polylinker and a unique XbaI site adjacent to the *ARS1* element, respectively. pEM209 was linearized using the two BamHI sites that flank the two *TEL* elements, thus producing a linear YAC plasmid with a *TEL* element at each end, and a 1768 bp spacer fragment with the yeast *HIS3* gene (Fig. 1).

Effects on Transformation Efficiency and Marker Stability. Transformation of moss generates many transient transformants that quickly lose the selection marker (12). As shown in Table S1, we found that transformation with circular DNA at first generates 2–4 times more transformants than linearized DNA. However, after 8 weeks of selection, only 0.4% of the transformants were alive, indicating that they could replicate and express the *npt II* cassette, and 87% of them were obtained with linearized DNA. Most of them were still alive after one year of selection (Table

Author contributions: E.M., A.N., and H.R. designed research; E.M., A.N., M.U., and M.J. performed research; E.M., A.N., M.U., M.J., and H.R. analyzed data; and H.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]E.M. and A.N. contributed equally to this work.

[2]Present address: Department of Food Science, Swedish University of Agricultural Sciences, Box 7051, SE-750 07 Uppsala, Sweden.

[3]To whom correspondence should be addressed. E-mail: hans.ronne@imbim.uu.se.

This article contains supporting information online at www.pnas.org/cgi/content/full/0908037106/DCSupplemental.

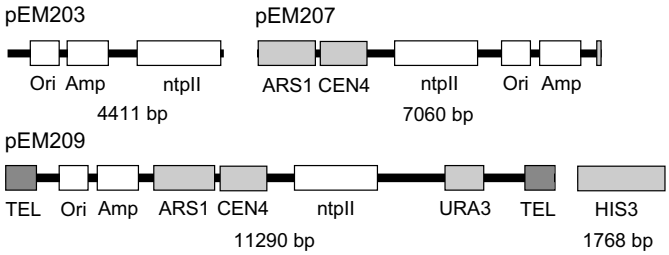


Fig. 1. Linear maps of plasmids used in the experiments. The endpoints are the sites at which linearized plasmids were cut before transformation.

S1). We conclude that linearized DNA is more efficient in generating nontransient transformants, which is consistent with previous observations (12–15, 17, 18). To distinguish between stable integrants and unstable (episomal) transformants, we used the method used to screen for integrants during gene targeting (7). We found that 50 of 76 transformants were stable, indicating that they were chromosomal integrants (Table S1).

One aim of our experiment was to investigate if the *ARS1*, *CEN4*, or *TEL* elements would affect transformation efficiency or marker stability. We did not see any striking effects that could be attributed to these elements. However, pEM207 and pEM209 did produce 2–4 times more nontransient transformants than pEM203 (Table S1), which suggests that these plasmids, which contain the *ARS1* and *CEN4* elements, are somewhat more stable in moss. Furthermore, integrants were enriched among linearized pEM209 transformants (21/24), as compared to all linearized transformants (43/67). The reason for this remains to be determined, but we note that the moss genome (10) contains several simple repeat regions that show extensive similarity to the *TEL* repeat. It is conceivable that this could facilitate integration of linearized pEM209 by homologous recombination.

Structure and Copy Number of the Transformed DNA. We selected 15 moss transformants for further studies, 1 obtained with circular DNA and 4 obtained with linearized DNA for each plasmid (Table 1). Of these 15 transformants, 8 scored as stable and 6 as unstable in both gametophore rescue (Fig. 2*A*) and plasmid loss experiments (Table 1 and Fig. 2*B*). One transformant, 203-L3,

Table 1. Moss transformants that were studied by plasmid rescue

Moss line	Type	Plasmid loss, %	Copy number	Rescued plasmids
203-C1	Unstable	84	15	19
203-L1	Stable	0	2	1
203-L2	Unstable	61	2430	645
203-L3	Semi-stable	2	10	7
203-L4	Stable	0	4	7
207-C1	Unstable	93	26	12
207-L1	Unstable	100	1750	210
207-L2	Unstable	81	2490	276
207-L3	Stable	0	64	13
207-L4	Unstable	100	2520	393
209-C1	Stable	0	2	0
209-L1	Stable	0	4	1
209-L2	Stable	0	6	1
209-L3	Stable	0	4	0
209-L4	Stable	0	3	1

In the names of the moss lines, C stands for transformants obtained with circular DNA and L for those obtained with linearized DNA. The numbers of rescued plasmids refer to a set of experiments where all 15 transformants were compared. More plasmids were subsequently rescued and characterized from transformants of particular interest (Table 2).

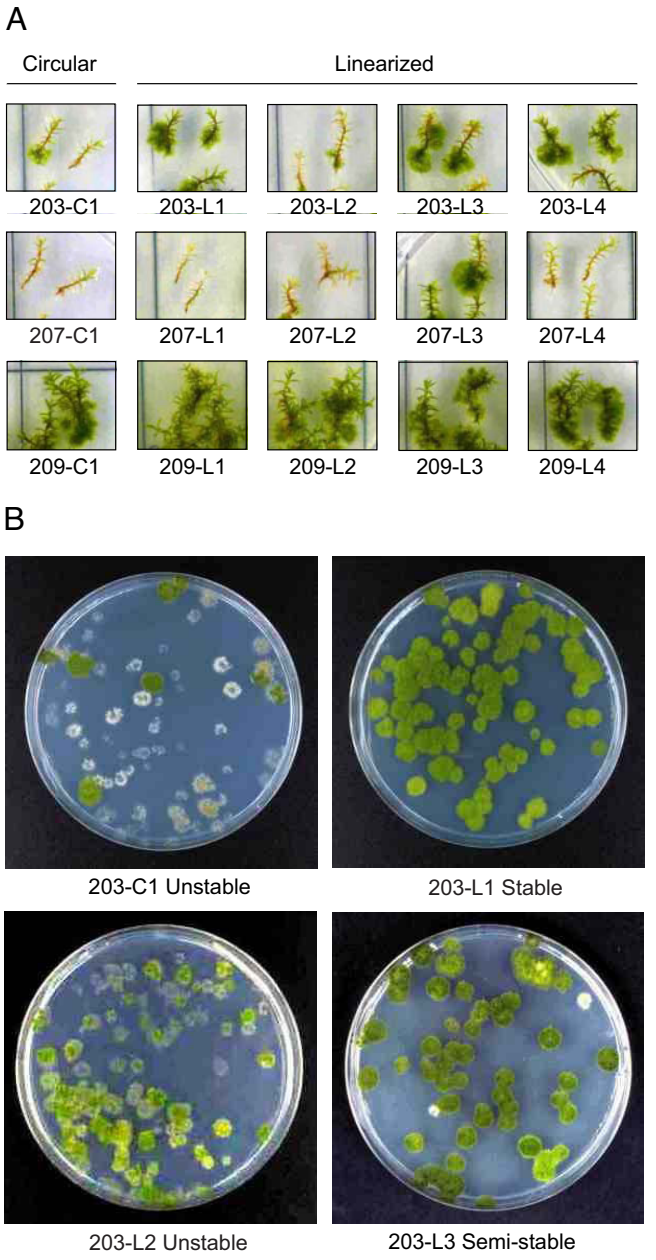


Fig. 2. Tests for stability of moss transformants. (*A*) Gametophore rescue. Only those gametophores that retained the plasmid are able to regenerate protonemal filaments when placed horizontally on selective plates. The 15 transformants in Table 1 are shown. (*B*) Plasmid loss experiment. Colonies that survived on selective plates due to retention of the plasmid are green, and those that lost the plasmid and died are white. Two unstable episomal transformants (203-C1 and 203-L2), one stable integrant (203-L1) and the semistable transformant (203-L3) are shown.

was semistable. It scored as stable in gametophore rescue (Fig. 2*A*), but the plasmid loss experiment (Fig. 2*B*) revealed that the marker was lost at a low but detectable frequency (2%) in the absence of selection. We proceeded to do Southern blots with undigested DNA from these 15 transformants and a probe from the *npt II* cassette. Consistent with previous observations (14, 15) we found that most of the hybridizing DNA migrated with low mobility or remained in the well, indicating a size of more than 10 kbp (Fig. 3*A*). In the case of stable transformants (integrants), this is the expected result of random shearing of chromosomal DNA. For unstable transformants, which have episomally rep-

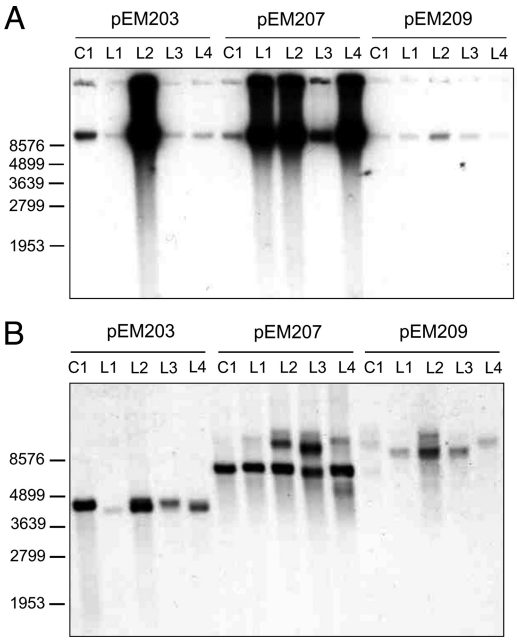


Fig. 3. Southern blots with DNA from the 15 transformants in Table 1. (*A*) Undigested DNA. The same amount of DNA (0.5 μg) was loaded from each transformant. The upper band is DNA that remained in the well. (*B*) DNA cut with enzymes that cut once in the respective plasmids. The amount of DNA (0.1–4 μg) was adjusted to make the signals more comparable in strength. The filters were hybridized to a HindIII fragment from the *npt II* marker, which is present on all 3 plasmids.

licating DNA, it suggests that most of the episomal DNA is present in high molecular weight complexes. The signal was also much stronger in the 4 unstable transformants obtained with linearized DNA, indicating a massive amplification of the transformed DNA in that case.

Restriction of the moss DNA with enzymes that cut once in each plasmid produced a major plasmid-sized band in most transformants, consistent with the presence of concatemeric head-to-tail arrays (Fig. 3*B*). The 3 integrants 209-L1, L2, and L3 produced a smaller band, since these integrants all share a 2432 bp deletion (Table 2). In some transformants, there was also hybridization to high molecular weight DNA, which suggests that some DNA is present in more complex structures than head-to-tail arrays. Copy numbers were estimated using Southern blots with an *npt II* probe (see *SI Text*). We found that integrants had between 2 and 64 copies of the *npt II* cassette (Table 1). The 2 episomal transformants obtained with circular DNA had 15 and 26 copies, whereas the 4 episomal transformants obtained with linearized DNA had between 1750 and 2520 copies. The semi-stable transformant 203-L3, finally, had approximately 10 copies.

Rescue of Plasmids Back to *E. coli* from Undigested Moss DNA. We proceeded to test if plasmids could be rescued back into *E. coli*. For each transformant, undigested DNA was used to transform *E. coli* cells after which ampicillin-resistant colonies were selected. Surprisingly, we could rescue plasmids from all moss transformants, including the integrants. No transformants were obtained with DNA from untransformed moss. Our finding that plasmids could be rescued also from the integrants raised the question whether these also contain episomally replicating DNA or whether these plasmids were created later, in *E. coli*. We reasoned that sheared chromosomal moss DNA with tandem copies of an integrated plasmid might circularize by homologous recombination in *E. coli* to produce circular plasmids. The *E. coli* strain that we used at first, SF8, is *recA*$^+$ and can thus carry out homologous recombination. We therefore repeated our experiment using the *recA*$^-$ *E. coli* strain TOP10F′. We found that this significantly reduced the number of plasmids that could be rescued from integrants; in most cases no plasmid or only a single plasmid was recovered. In contrast, we could easily rescue plasmids from unstable moss transformants containing episomally replicating DNA (Table 1).

Table 2. Rescued plasmids that were further characterized

Moss line	Type	Wild type plasmids	Deletion plasmids	Length of deletions, bp
203-C1	Unstable	76	0	—
203-L1	Stable	2	16	260
203-L2	Unstable	12	16	260
203-L3	Semi-stable	17	7	260
203-L4	Stable	5	14	260
207-C1	Unstable	19	0	—
207-L1	Unstable	10	2	71
207-L2	Unstable	20	0	—
207-L3	Stable	2	10	1192, 981, 462, 199
207-L4	Unstable	0	14	3838, 49, 48, 46
209-C1	Stable	2	1	168
209-L1	Stable	0	2	2432
209-L2	Stable	0	6	2432
209-L3	Stable	0	6	2432
209-L4	Stable	1	0	—

In the names of the moss lines, C stands for transformants obtained with circular DNA and L for those obtained with linearized DNA. When only one deletion is listed, all deletion plasmids rescued from that moss transformant had the same deletion.

Faithful Recovery of the Original Plasmid from Circular DNA Transformants. In total, 260 rescued plasmids were characterized by restriction mapping, and 61 plasmids were sequenced. As shown in Table 2, all but one of the 98 plasmids that were rescued from transformants obtained with circular DNA were identical to the original plasmid. The one exception was a plasmid rescued from 209-C1, which had undergone recombination within 1 of the 2 *TEL* arrays, losing 28 repeats. It should be noted that the *TEL* arrays provide much longer targets for homologous recombination than is present in the other plasmids, which may explain why such an event was observed in this case. The fact that we did not recover any other plasmids with deletions or rearrangements suggests that such events are rare after transformation with circular DNA. It does not necessarily mean that the transformed DNA replicates as monomeric circles in moss. As shown in Fig. 3*A*, the DNA forms high molecular weight structures also in the episomal transformants. However, the absence of deletions and rearrangements suggests that the episomal DNA is formed by recombination between linear or circular monomers, creating simple head-to-tail concatemers. Consistent with this, these transformants produced a single major band in Southern blots with an enzyme that cuts the plasmid once (Fig. 3*B*).

Linearized DNA Is Repaired in Moss Either by Religation or by Recombination. For moss transformants obtained with linearized DNA, the results were more complicated. With the smallest plasmid, pEM203, all 4 transformants studied produced 2 types of plasmids, one of which was identical to pEM203. The BamHI site used to linearize the DNA before transformation was present in these plasmids, suggesting that a precise religation had occurred. This was confirmed by sequencing of several plasmids. The other type of plasmids that were rescued from these transformants were all identical to each other, with a 260 bp deletion spanning the 3′-untranslated part of the *npt II* cassette. This deletion is flanked by a 17 bp repeat derived from the

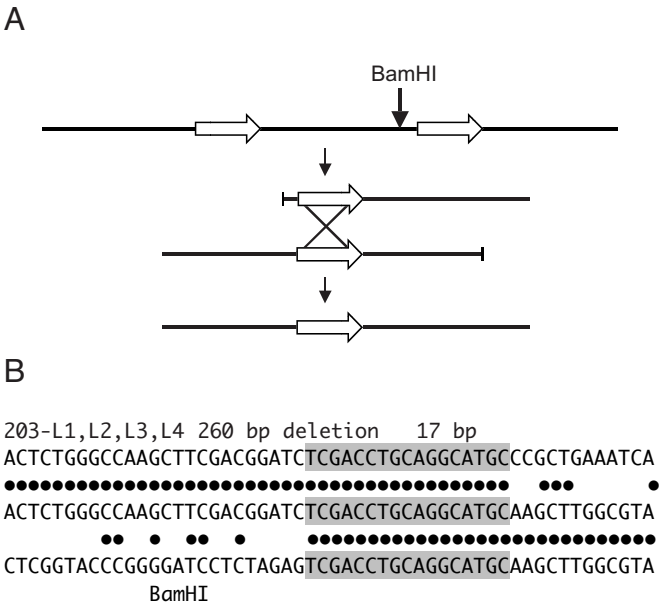


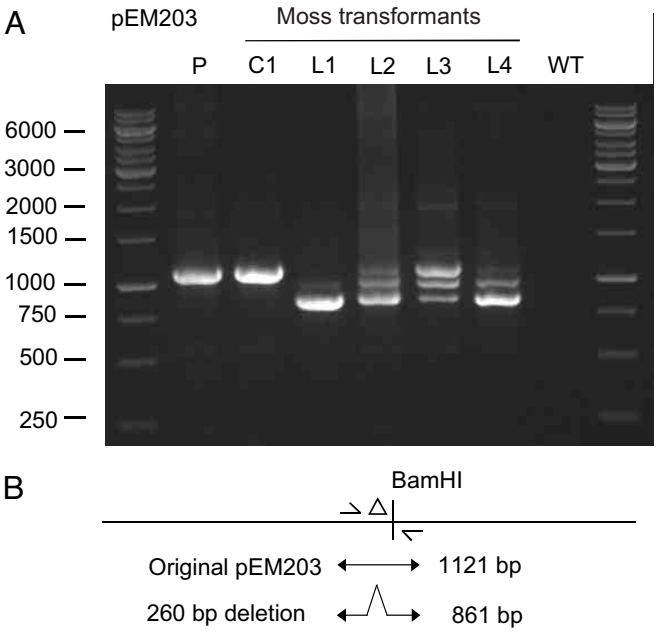
Fig. 4. Creation of the 260 bp deletion by recombinational repair of linearized pEM203 in moss. (*A*) Schematic picture of the repair process. (*B*) Alignment of parental and recombinant pEM203 sequences. The 17 bp direct repeats are enclosed in gray boxes. The BamHI site used to linearize pEM203 is shown below the bottom sequence.

pUC19 polylinker, which is fortuitously present twice in pEM203, and the deletion was created by recombination between these 2 repeats (Fig. 4).

Significantly, both plasmids were recovered from all 4 transformants obtained with linearized pEM203. To confirm that a mixture of both plasmids were present in each moss transformant and that the deletions were not created after rescue into *E. coli*, we carried out a PCR on moss DNA with primers that amplify the region spanning the deletion (Fig. 5). As expected, we observed bands corresponding to both intact pEM203 (1121 bp) and the 260 bp deletion (861 bp) in the transformants obtained with linearized pEM203 (a third intermediate band is most likely a heteroduplex produced during the final annealing step). In contrast, the transformant obtained with circular pEM203 DNA, 203-C1, produced only the 1121 bp band, consistent with the fact that we could rescue only wild-type plasmids from this transformant.

To verify the identity of the 2 bands, we cloned and sequenced a number of PCR fragments amplified from moss DNA. As shown in Fig. 5*C*, 3 of the 4 transformants obtained with linearized DNA yielded both types of sequences (original pEM203 and the 260 bp deletion) whereas the circular transformant yielded only the wild type sequence. We conclude that the same 260 bp deletion was created in several independent moss transformants, all of which also retained the original plasmid. This suggests that recombination between the 17 bp direct repeats occur at high frequency after transformation of linearized pEM203 into moss. However, our recovery of the original plasmid from all moss transformants shows that such processing is not required and suggests that cohesive end ligation also occurs at a high frequency.

Multiple Repair Events Can Occur in the Same Moss Transformant. For pEM207, we used an XbaI site to linearize the plasmid before transformation into moss (Fig. 1). All plasmids rescued from transformant 207-L2 had retained the XbaI site. From 207-L1 and 207-L3, we could rescue both plasmids with and without the XbaI site. From 207-L4, we only recovered plasmids that had lost



C

Cloned PCR products	C1	L1	L2	L3	L4
Original pEM203	3	0	1	2	1
260 bp deletion	0	2	2	2	2

Fig. 5. PCR amplification of plasmid head-to-tail junction fragments from different pEM203 transformants. (*A*) Agarose gel with PCR products. P stands for the original pEM203 plasmid and WT for DNA from wild-type moss. (*B*) Partial map of 2 head-to-tail copies of the plasmid, with PCR primers and expected PCR fragments shown. (*C*) The number of cloned PCR products from each transformant that had the original pEM203 sequence or the 260 bp deletion variant.

the site. Sequencing revealed that plasmids without XbaI sites had deletions ranging from 46 to 3838 bp in size (Table 2). All deletions except one were created by direct repeat recombination between short (2–8 bp) repeats (Fig. S1). The results were thus similar to those obtained with linearized pEM203 in that both the original plasmid and deletion variants could be rescued from most transformants. There was, however, much more variety in the deletions. A likely explanation is that the recombination machinery prefers longer repeats, like the 17 bp repeat in pEM203, whereas the longest direct repeat in pEM207 is only 11 bp.

No less than 4 different deletions, as well as intact pEM207, could be rescued from 207-L3, the only stable moss transformant obtained with pEM207. Furthermore, the PCR yielded a smear in addition to the expected band, indicating a more complex structure for the integrated DNA (Fig. S2). Consistent with this, PCRs with only one primer, which can detect head-to-head or tail-to-tail repeats, yielded distinct bands with DNA from 207-L3 but not with any other transformant (Fig. S3). We conclude that multiple independent recombination events, as well as the creation of head-to-tail, head-to-head, and tail-to-tail repeats, can occur in the same moss transformant. However, the fact that we saw evidence of this in only 1 of our 15 transformants suggests that it is not very common.

A somewhat different result was obtained with the unstable transformant 207-L4, from which 3 plasmids with very similar but nonidentical deletions of 46, 48, and 49 bp could be rescued.

GENETICS

The 46 bp deletion was also associated with two adjacent point mutations (Fig. S1). A likely interpretation is that the 3 deletions arose by imperfect amplifications of the same original deletion within one or more episomal arrays. One plasmid rescued from 207-L4 had a large deletion of 3838 bp, but the left endpoint of this deletion was the same as in the smaller deletions (Fig. S1), which suggests that it could have arisen in a secondary event from the latter.

A 12 bp Repeat Is Sufficient for Preferential Targeting of Recombination in Moss. For the pEM209 transformants, we first tested if rescued plasmids could be cut with BamHI, which was used to linearize pEM209 before transformation (Fig. 1). We found only one plasmid that could be cut with BamHI, and this plasmid had in fact retained both BamHI sites and the internal *HIS3* fragment of pEM209. This suggests that it may have been derived from an uncut molecule. All 14 plasmids rescued from the remaining 3 pEM209 transformants obtained with linearized DNA had the same 2432 bp deletion (Table 2). This deletion was created by recombination between two 12 bp repeats (Fig. S4), which is the longest direct repeat in pEM209 (the 2 *TEL* arrays are longer, but in inverted orientation relative to each other). This supports the notion that the recombination machinery picks the longest direct repeat available to repair free DNA ends and further suggests that 12 bp may be the minimal length required for such preferential targeting. Finally, 1 deletion was associated with 2 point mutations (Fig. S4), similar to what was seen for pEM207. This suggests that direct repeat recombination is mutagenic in moss.

Discussion

Previous work has shown that *Physcomitrella* can replicate foreign DNA episomally (12–15). Here we show that such foreign DNA can be rescued back into *E. coli* from undigested moss DNA and characterize the resulting plasmids. Several conclusions can be drawn from our results about the fate of transformed DNA in *Physcomitrella*.

A striking finding is the lack of rearrangements, deletions, or insertions of host DNA in plasmids rescued from episomal (unstable) moss transformants obtained with circular plasmid DNA. The 95 rescued plasmids were in all cases identical to the original pEM203 or pEM207 plasmid (Table 2). It shows that moss is capable of faithful episomal replication of foreign DNA without rearrangements. This is unlike episomal vectors in mammalian cells, where rearrangements and insertions of host DNA are frequent (19), and instead resembles yeast shuttle vectors. It does not rule out that replication in moss involves complex intermediates, such as episomal concatemers (15), since the latter could pop out circles by recombination (Fig. 6). The same is true for chromosomally integrated concatemers, which also could pop out circular plasmids (Fig. 6). An example of this from yeast is the circular ERC molecules which pop out from rDNA arrays (20). In fact, popouts of circles from chromosomal arrays may explain why plasmids occasionally could be rescued also from our stable transformants (Table 1). Regardless of the mode of replication, the fact that the input plasmid easily can be recovered without rearrangements is significant, since it makes molecular methods that rely on this, such as cloning by complementation and dosage suppressor screens, possible.

Linearization of the DNA significantly increased the number of moss transformants (Table S1). Plasmids rescued from these transformants were either identical to the original plasmid or had deletions in which the site used to linearize the DNA had been lost (Table 2). Both types of plasmids, and sometimes several different deletions, could be rescued from the same moss transformants, and PCRs confirmed that some transformants harbored a mixed population of plasmids (Fig. 5). Interestingly, we found that the deletions in all but one case involved recombination between short (2–17 bp) direct repeats (Figs. 4, S1, and S4). This illustrates how efficient recombination is in moss and further suggests that recombination has a strong bias for the longest region of similarity, a finding which is consistent with studies of gene targeting in moss (9, 17, 18). Thus, all deletions recovered from linearized pEM203 involved the same 17 bp repeat, the longest repeat in pEM203, and all deletions recovered from linearized pEM209 transformants involved the same 12 bp repeat, the longest repeat in that plasmid. In contrast, linearized pEM207, which only has repeats of 11 bp or less, produced several different deletions. This suggests that 12 bp may be the minimal length needed for preferential targeting of recombination in moss.

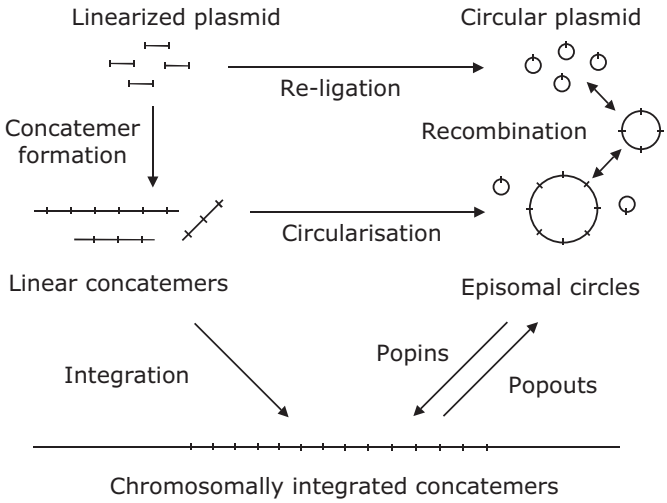


Fig. 6. Different possible fates of circular and linearized plasmid DNA after transformation into moss cells.

Our study confirmed that some episomal transformants are semistable, with the marker being lost at a much lower frequency (14, 15). It has been suggested that such transformants may carry moss DNA within the concatemers, perhaps derived from cells that lysed during transformation, which could promote replication or stability (14). However, plasmids rescued from the semistable transformant 203-L3 contained no moss DNA, and PCRs yielded only the products expected from simple head-to-tail arrays (Fig. 5). An alternative explanation for increased stability of some episomal transformants would be a higher copy number, but we saw no evidence of this either. On the contrary, episomal transformants with high copy numbers were very unstable, whereas the semistable 203-L3 only had a few copies (Table 1). We conclude that high copy number does not guarantee stable replication in moss. This is similar to yeast, where *ARS* plasmids, which exist in 20–50 copies per cell, still are highly unstable due to preferential segregation to mother cells (21). It is only by including a yeast centromere or DNA from the 2 μm plasmid, which has evolved a system for amplification that maintains a stable copy number, that stable replication can be achieved in yeast.

We think that a more likely explanation for the semistable transformants could be integration into one of the accessory minichromosomes that are present in mosses (22). A nonessential accessory minichromosome could provide a vehicle for propagation of foreign DNA, which is comparatively stable but still subject to occasional loss, similar to a *CEN* plasmid in yeast. Furthermore, the instability of most episomal transformants, even those with a very high copy number (Table 1), suggests that a segregation bias may exist also in moss. We note that cell division in moss filaments, which takes place at the tip (23), is

polarized, with significant differences between basal and tip cells. If episomal DNA segregates preferentially to the basal cell (or the tip cell) this could explain the high frequency of plasmid loss, similar to the effect of preferential segregation of *ARS* plasmids to yeast mother cells (21).

The usefulness of shuttle plasmids in yeast depends on 3 important features: (*i*) the ability to replicate foreign DNA in high copy number, which permits dosage suppressor screens, (*ii*) easy rescue of the original plasmid back into *E. coli* without rearrangements or insertions of host DNA, which greatly facilitates molecular genetics work, and (*iii*) the feasibility of plasmid loss experiments, which makes it easy to prove that a phenotype is plasmid-dependent. Our results show that all 3 features are available in moss. High copy numbers can be achieved for episomal transformants, and the original plasmid can be rescued back into *E. coli* without rearrangements from moss transformants obtained with circular DNA. A potential drawback of using circular DNA for transformation of moss is the lower efficiency as compared to linearized DNA (Table S1). However, we found that those deletions which occur in linearized DNA are highly predictable since they involve the longest direct repeat on a plasmid (Fig. 4). By using a vector similar to pEM203, in which sufficiently long direct repeats flank the site used to linearize the DNA, it is thus possible to limit the processing in moss to a predictable small deletion which does not affect the experiment. Finally, loss of a plasmid encoded phenotype is easy to score in plasmid loss experiments (Fig. 2). We conclude that episomally replicating plasmids that can be rescued back into *E. coli* provide the necessary tools for using shuttle plasmid-based methods in moss.

Materials and Methods

Transformation and Cultivation of Moss. The methods used for moss cultivation and transformation have been described (24). Aliquots of 20 μg of DNA were used to transform 4×10^5 protoplasts. Protoplasts were plated on cellophane-covered regeneration plates and then transferred after 5 days to BCD plates containing 5 mM ammonium tartrate (MM plates) and 50 μg/ml G418. Large colonies were picked after 3–5 weeks to fresh selection plates. These colonies were further subcultivated every 2 weeks by transferring small pieces of protonemal tissue to new selection plates.

Plasmid Stability Assays. To score plasmid stability in Table S1, we used the method used to screen for integrants during gene targeting (7). Pieces of protonemal tissue from transformants grown on selective media for 9 weeks were moved to nonselective plates for 5 weeks. A piece from each colony was then moved back to a selective plate, and transformants that survived after 2.5 weeks were scored as stable. For the gametophore rescue experiment (14) in Fig. 2*A*, colonies from 11-weeks-old transformants grown on selective plates were moved to nonselective plates and incubated for 3 weeks. Several gametophores were then dissected from each colony and placed on selective plates. Pictures were taken in an Olympus SZX12 dissecting microscope after 2 weeks, when new protonemal filaments had regenerated from the gametophores. For the plasmid loss experiments in Table 1 and Fig. 2*B*, tissue from colonies grown on selective plates for 11 months was homogenized in a Mini Beadbeater-8 (Biospec) and plated on nonselective cellophane covered plates. After 5 days, the tissue was collected, fragmented in an Omni Mixer homogenizer, diluted to generate an estimated 100 colonies per plate, and plated on cellophane overlaid nonselective plates. After 12 days, the cellophane sheets were moved to selective plates and incubated for 7 days. The numbers of surviving (green) and dead (white) colonies were then counted.

Molecular Genetics Methods. Plasmid construction and methods used for plasmid rescue, PCR experiments and Southern blots are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Neil Ashton, David Cove, Celia Knight, Didier Schaefer, Mattias Thelander, and Gerhart Wagner for helpful discussions. This work was funded by grants from the Swedish Research Council Formas, the Swedish Foundation for Strategic Research, and the Uppsala Centre for Functional Genomics.

1. Orr-Weaver TL, Szostak JW, Rothstein RJ (1981) Yeast transformation: A model system for the study of recombination. *Proc Natl Acad Sci USA* 78:6354–6358.
2. Beggs JD (1978) Transformation of yeast by a replicating hybrid plasmid. *Nature* 275:104–109.
3. Stinchcomb DT, Struhl K, Davis RW (1979) Isolation and characterisation of a yeast chromosomal replicator. *Nature* 282:39–43.
4. Clarke L, Carbon J (1980) Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature* 287:504–509.
5. Struhl K (1983) The new yeast genetics. *Nature* 305:391–397.
6. Rine J (1991) Gene overexpression studies of *Saccharomyces cerevisiae*. *Methods Enzymol* 194:239–251.
7. Schaefer DG, Zrÿd J-P (1997) Efficient gene targeting in the moss *Physcomitrella*. *Plant J* 11:1195–1206.
8. Schaefer DG (2002) A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. *Annu Rev Plant Biol* 53:477–501.
9. Cove D (2005) The moss *Physcomitrella patens*. *Annu Rev Genet* 39:339–358.
10. Rensing SA, et al. (2008) The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319:64–69.
11. Lang D, Zimmer AD, Rensing SA, Reski R (2008) Exploring plant biodiversity: The *Physcomitrella* genome and beyond. *Trends Plants Sci* 13:542–549.
12. Schaefer DG, Zrÿd J-P, Knight CD, Cove DJ (1991) Stable transformation of the moss *Physcomitrella patens*. *Mol Gen Genet* 226:418–424.
13. Knight CD (1994) Studying plant development in mosses: The transgenic route. *Plant Cell Environ* 17:669–674.
14. Schaefer DG (1994) Molecular genetic approaches to the biology of the moss *Physcomitrella patens*. PhD dissertation (Université de Lausanne, Lausanne, Switzerland).
15. Ashton NW, Champagne CEM, Weiler T, Verkoczy LK (2000) The bryophyte *Physcomitrella patens* replicates extrachromosomal transgenic elements. *New Phytol* 146:391–402.
16. Burke DT, Carle GF, Olson MV (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* 236:806–812.
17. Kamisugi Y, et al. (2006) The mechanism of gene targeting in *Physcomitrella patens*: Homologous recombination, concatenation, and multiple integration. *Nucl Acids Res* 34:6205–6214.
18. Kamisugi Y, Cuming AC, Cove DJ (2005) Parameters determining the efficiency of gene targeting in the moss *Physcomitrella patens*. *Nucleic Acids Res* 33:e173.
19. Van Craenerbroeck K, Vanhoenacker P, Haegeman G (2000) Episomal vectors for gene expression in mammalian cells. *Eur J Biochem* 267:5665–5678.
20. Sinclair DA, Guarente L (1997) Extrachromosomal rDNA circles—a cause of aging in yeast. *Cell* 91:1033–1042.
21. Murray AW, Szostak JW (1983) Pedigree analysis of plasmid segregation in yeast. *Cell* 34:961–970.
22. Newton ME (1984) The cytogenetics of bryophytes. In The Experimental Biology of Bryophytes, eds Dyaer AF, Duckett JG (Academic Press, London), pp 65–96.
23. Menand B, Calder G, Dolan L (2007) Both chloronemal and caulonemal cells expand by tip growth in the moss *Physcomitrella patens*. *J Exp Bot* 58:1843–1849.
24. Thelander M, et al. (2007) The moss genes PpSKI1 and PpSKI2 encode nuclear SnRK1 interacting proteins with homologues in vascular plants. *Plant Mol Biol* 64:559–573.