Plant Cell Rep (2002) 21:341–346
DOI 10.1007/s00299-002-0521-5

GENETIC TRANSFORMATION AND HYBRIDIZATION

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Promoter subfragments of the sugar beet V-type H$^+$-ATPase subunit c isoform drive the expression of transgenes in the moss *Physcomitrella patens*

Received: 24 April 2002 / Revised: 1 August 2002 / Accepted: 6 August 2002 / Published online: 20 September 2002

Abstract Based on the relative ease of performing targeted nuclear gene knockout, the moss *Physcomitrella patens* has recently been developed as a model system for plant functional genomics. To address the need for new promoters that could drive expression of transgenes in this moss, we tested two fragments of the promoter region of the gene for the sugar beet (*Beta vulgaris*) V-type H$^+$-ATPase subunit isoform c. Four gene knockout constructs were tested in which the neomycin phosphotransferase II selection marker gene was put under the control of two distinct V-type H$^+$-ATPase promoter fragments, the NOS promoter, or the CaMV 35S promoter. In each case the selection cassettes were flanked by moss FtsZ1 cDNA sequences to facilitate chromosomal targeting. From a total of more than 440 transformed plants, the number of plants generated per construct was monitored and found to be in the range of 5 to 11 stable transgenics per transformation. Both V-type H$^+$-ATPase promoter fragments lead to NPTII expression levels that were sufficiently high to generate large numbers of stable transgenic plants. The numbers of plants obtained with the two V-type H$^+$-ATPase promoter fragments were comparable to those with constructs containing the standard NOS and 35S promoters. We propose that the higher plant V-type H$^+$-ATPase promoter can be used for the expression of transgenes in the bryophyte *P. patens*.

Keywords Functional genomics · V-ATPase, Promoter · *Physcomitrella*

Abbreviations *CaMV*: Cauliflower mosaic virus · *NOS*: Nopaline synthase · *NPTII*: Neomycin phosphotransferase II

Communicated by H. Lörz

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Introduction

The moss *Physcomitrella* has recently been demonstrated to allow rapid identification of plant gene function by virtue of targeted gene knockout (Schaefer and Zryd 1997; Girke et al. 1998; Reski 1998; Strepp et al. 1998; Girod et al. 1999; Reski 1999; Nishiyama et al. 2000; Schaefer 2001). Generally, the use of transgenic technology is dependent on an array of molecular genetic tools among which suitable selectable marker genes and sufficiently strong promoters are the most important (Holtorf et al. 2002). The fact that only a few promoter sequences of moss genes are currently available and that *Physcomitrella* exhibits a high rate of homologous recombination (Reski 1998, 1999) urges moss researchers to use heterologous promoters for the expression of selection-marker genes and other genes of interest. However, only a few such promoters have been reported to function reliably in *Physcomitrella*. Among the promoters analysed are the cauliflower mosaic virus (CaMV) 35S promoter (Benfey and Chua 1990), the *Agrobacterium* nopaline synthase (*nos*) (Fraley et al. 1983), *ipt* (Akiyoshi et al. 1984), and 1′ (Kammerer and Cove 1996) promoters, the rice actin Act1 promoter (McElroy et al. 1991), a soybean heat-shock promoter (Schoeffl et al. 1989), the *em* promoter from wheat (Knight et al. 1995), the *rbcS* promoter from *Chlamydomonas* (Kozminski et al. 1993), and the artificial TOP10 promoter (Weinmann et al. 1994). In *Physcomitrella*, these promoters have been demonstrated to drive the expression of the widely used plant select-

Table 1 Promoter gene fusions in *Physcomitrella patens*. *UidA* β-glucuronidase (GUS), *gfp* green fluorescent protein, *nptII* neomycin phosphotransferase II, *hpt* hygromycin phosphotransferase, *ipt* isopentenyl phosphotransferase, *NOS* nopaline synthase

Promoter	Source	Gene	Transformation Stable/transient	Reference
NOS	*Agrobacterium tumefaciens*	*nptII*	Stable	Reutter et al. (1998) Egener et al. (2002)
IPT	*A. tumefaciens*	*ipt*	Stable	Reutter et al. (1998)
1′	*A. tumefaciens*	*hpt*	Stable	Kammerer and Cove (1996)
CaMV 35S	Cauliflower mosaic virus	*hpt*	Stable	Girod et al. (1999)
		nptII	Stable	Strepp et al. (1998) Nishiyama et al. (2000)
		gfp	Transient	Kiessling et al. (2000)
		uidA	Transient	Zeidler et al. (1999)
Rice actin Act1	*Oryza sativa*	*uidA*	Transient	Chakhparonian et al. (2000) Zeidler et al. (1999)
		gfp	Stable	Nishiyama and Hasebe (2001)
Heat shock	*Glycine max*	*uidA*	Transient	Zeidler et al. (1999)
EM	*Triticum aestivum*	*uidA*	Stable/transient	Knight et al. (1995)
RbcS	*Chlamydomonas reinhardtii*	*uidA*	Transient	Zeidler et al. (1999)
TOP10	artificial	*uidA*	Stable	Zeidler et al. (1996)

able-marker genes *nptII* (Reutter et al. 1998; Strepp et al. 1998; Egener et al. 2002) and *hpt* (Girod et al. 1999), as well as the reporter genes β-glucuronidase (GUS) (*uidA*; Knight et al. 1995; Zeidler et al. 1996; Chakhparonian et al. 2000) and green fluorescent protein (*gfp*; Kiessling et al. 2000; Nishiyama and Hasebe 2001) (Table 1). When comparing different promoter-*uidA* constructs, reporter gene expression levels showed marked differences in *Physcomitrella* and single promoters varied in strength between the two moss species *Physcomitrella* and *Ceratodon* (Zeidler et al. 1996, 1999). In general, experimental systems involving various gene cassettes necessitate different promoter sequences to minimise molecular interactions by homologous recombination processes between the different constructs. Therefore, it is very important to identify sufficiently strong heterologous promoters for the expression of transgenes in *Physcomitrella*.

We investigated if expression signals from higher plants can be used to drive expression of heterologous genes in *Physcomitrella*. In this study, we tested if the promoter sequence BVA/16–1 of the sugar beet V-type H^+-ATPase subunit c isoform gene (Lehr et al. 1999) could serve to express selection marker genes in moss plants. The expression signals of BVA/16–1 were chosen as a promising candidate promoter because reporter gene assays measuring luciferase (LUC) reporter gene activity demonstrated that the BVA/16–1 promoter strength in suspension-cultured cells of sugar beet is comparable to that of the CaMV 35S promoter (Lehr et al. 1999). Two distinct BVA/16–1 promoter fragments, the near complete promoter DelA (992 bp) and a subfragment DelB (242 bp) including the transcriptional start site, were tested for their ability to drive expression of the standard selectable marker gene nptII in transgenic *Physcomitrella* plants using a high throughput transformation platform in our laboratory (Egener et al. 2002). Performance of the BVA/16–1 promoters was compared with both the *NOS* and CaMV 35S promoters. The number of stable transgenic plants generated using either the DelA or DelB construct was in the same range as the output of transformants obtained with constructs containing the CaMV 35S or *NOS* expression signals.

Materials and methods

Plant material, protoplast isolation and transformation

The moss *Physcomitrella patens* (Hedw.) B.S.G. was propagated as suspension culture in modified Knop medium containing (per litre) 1,000 mg $Ca(NO_3)_2 \cdot 4H_2O$, 250 mg KCl, 250 mg KH_2PO_4, 250 mg $MgSO_4 \cdot 7H_2O$ and 12.5 mg $FeSO_4 \cdot 7H_2O$ (pH 5.8) as described (Reski and Abel 1985). In order to obtain high amounts of uniform plant material, semi-continuous bioreactor cultures were set up as described (Hohe and Reski 2002; Hohe et al. 2002). This material was used for protoplast isolation following a protocol adapted from Rother et al. (1994). The cell material of 200 ml suspension was digested in 16 ml 2% Driselase (Sigma, Taufkirchen, Germany) in 0.5 *M* mannitol for 45 min and afterwards passed through sieves with a mesh size of 100 and 45 µm, respectively. The resulting protoplast suspension was centrifuged for 10 min at 45 *g*, the pellet washed in 0.5 *M* mannitol and, after a second centrifugation, the number of protoplasts was determined by counting in a Fuchs-Rosenthal-chamber. Transformations were performed with 3×10^5 protoplasts suspended in 250 µl 3M medium (15 m*M* $MgCl_2$, 0.1% MES, 0.48 *M* mannitol, pH 5.6) mixed with 350 µl PEG solution (40% PEG 4,000 in 3M medium, pH 6.0) and DNA in 100 µl 0.1 *M* $Ca(NO_3)_2$ following the protocol described by Strepp et al. (1998). Unique *Eco*RI and *Ecl*136II restriction sites in the two FtsZ1 cDNA stretches adjacent to the selection cassette were used to linearising the knockout constructs prior to transformation. All transformations were performed in parallel. After 30 min incubation with occasional gentle mixing, the transformation solution was diluted with 3M medium every 5 min (adding 1, 2, 3 and 4 ml). After centrifugation (10 min, 45 *g*) the pellet was resuspended in regeneration medium (Knop medium as described above supplemented with 5% glucose and 3% mannitol, pH 5.6, 540 mOs) and transferred to 3 cm Petri dishes. The cultures were incubated for 1 day in the dark, subsequently at 70 µmol s^{-1} m^{-2} and 25°C. After 11 days the liquid cultures were plated on Knop medium as described above, solidified with 10 g/l agar, and covered with cellophane. After another 3 days the cellophane with the cultures was transferred to selection medium containing 25 µg/ml geniticin (G418, Promega, Germany) for 2 weeks. This was fol-

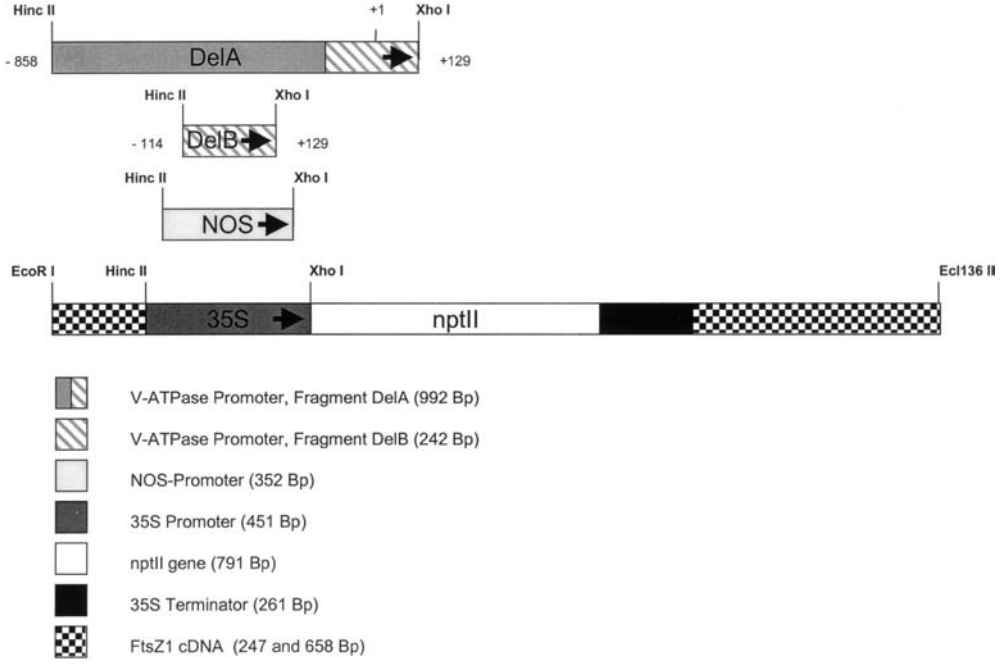

Fig. 1 Scheme showing the construction of plasmids Lig10/1-DelA, Lig10/1Del-B and Lig10/1-NOS by inserting the promoter fragments DelA, DelB, and NOS into the backbone of the FtsZ1 knockout construct Lig10/1. PCR-fragments of the promoters were generated using primers harbouring *Hinc*II and *Xho*I restriction sites. *Eco*RI and *Ecl*136II restriction sites were used for linearisation of the DNA prior to transformation. Relevant restriction sites, the transcriptional start site (+1), and the lengths of promoters in base pairs (bp) are indicated. The two FtsZ1 fragments that are situated 5′ and 3′ of the nptII selection cassette are 257 and 658 bp in length, respectively (FtsZ1; GenBank accession number AJ249138)

lowed by a 2 week growth cycle on medium without G418 and a second selection on medium with 25 µg/ml G418 for 2 weeks. Finally, the number of surviving plants was counted.

Construction of FtsZ1 knockout cassettes

Manipulation of nucleic acids was performed according to standard procedures (Ausubel et al. 1993). All constructions of new FtsZ1 knockout cassettes were based on the plasmid Lig10/1, which served as the knockout construct for the moss FtsZ1 gene (Strepp et al. 1998). The CaMV 35S promoter fragment (451 bp) in Lig10/1 was exchanged for either the NOS gene promoter (352 bp), the sugar beet V-type H^+-ATPase DelA (992 bp), or DelB (242 bp) promoter fragments, using the restriction sites *Hinc*II and *Xho*I. The DelA fragment comprises the main part of the BVA/16–1 promoter ranging from base –858 to +129 (Lehr et al. 1999; BVA/16–1: EMBL accession number Y11037). DelB is a subfragment of the BVA/16–1 promoter ranging from base –114 to +129. Prior to insertion of the DelA, DelB, and NOS fragments into Lig10/1, each promoter was amplified by polymerase chain reaction (PCR) from the plasmids pBVA/16–1 LUC (a gift from Ruth Viereck, University of Heidelberg), and pBSNNN (Egener et al. 2002), respectively, with primers harbouring *Hinc*II or *Xho*I restriction sites. Amplified and purified PCR fragments were digested with restriction enzymes *Hinc*II and *Xho*I and ligated into the *Hinc*II/*Xho*I-cut backbone of the Lig10/1 plasmid. Recombinant bacterial clones were analysed by restriction digests and agarose gel electrophoresis. The sequence and orientation of the inserted fragments in the recombinant clones was verified by sequencing with primers LigF (5′-CTTTCGCCTTTGAAGGGCGG-3′) and ATGR2 (5′-GAATGAACTGCAGGACGAGG-3′) reading from the 5′-oriented FtsZ1 cDNA and *nptII* coding sequences, respectively. The resulting plasmids were named Lig10/1-DelA, Lig10/1-DelB, and Lig10/1-NOS (Fig. 1). Equimolar amounts of plasmids (50 µg for constructs Lig10/1, Lig10/1-DelA, Lig10/1-NOS and 56 µg for Lig10/1-DelB) were used for transformation.

Suspension cultures

Beta vulgaris suspension-cultured cells were cultivated in 250 ml Erlenmeyer flasks and subcultured every 7 days by diluting 20 ml cell culture into 50 ml fresh Gamborg B5 medium complemented with 2% sucrose, 2 mg/l 2,4-D, 0.5 mg/l 1-NAA, 0.5 mg/l IAA, and 0.2 mg/l kinetin (pH adjusted to 5.5). Cell cultures were kept in the dark on a rotary shaker at 100 rpm. Prior to bombardment, 3-day-old cultures (1 ml packed cell volume/plate) were evenly spread on a 4.5 cm filter disk by vacuum filtration. Thereafter, the filters were layered on agar plates (0.9%), prepared with Gamborg B5 medium. Post bombardment, cells were kept in the dark for 20 h, after which cell extracts were prepared and assayed for LUC and GUS activity.

Heterotrophically growing *P. patens* suspension cultures were established from solid medium cultures by transfer to the following liquid medium: 1.84 m*M* KH_2PO_4, 3.36 m*M* KCl, 1.02 m*M* $MgSO_4 \cdot 7H_2O$, 4.24 m*M* $Ca(NO_3)_2 \cdot 4H_2O$, 45 µ*M* $FeSO_4 \cdot 7H_2O$, 50 µ*M* H_3BO_3, 50 µ*M* $MnSO_4 \cdot H_2O$, 15 µ*M* $ZnSO_4 \cdot 7H_2O$, 2.5 µ*M* KI, 0.5 µ*M* $Na_2MoO_4 \cdot 2H_2O$, 0.05 µ*M* $CuSO_4 \cdot 5H_2O$, 0.05 µ*M* $CoCl_2 \cdot 6H_2O$, supplemented with 3% glucose. As with sugar beet cells (see above), the moss suspension was transferred to 4.5 cm filters prior to bombardment.

Particle bombardment with promoter-LUC fusions

The V-ATPase subunit c promoter deletions DelA and DelB were prepared with the Exo Mung Bean Deletion Kit (Stratagene) from the full length subunit c promoter-LUC fusion described in Lehr et al. (1999). For bombardment, the DelA/B LUC constructs were mixed at a ratio of 7:3 (w/w) with the calibration plasmid pFF19G, containing the GUS gene under the control of an enhanced 35S promoter (Lehr et al. 1999). Particle bombardment and reporter gene assays were performed as in Lehr et al. (1999). By measuring LUC and GUS activities in the same extract, LUC activities were calibrated for equal transformation efficiency obtained from the GUS activities.

Polymerase chain reaction

DNA of plasmids pBVA/16–1 LUC and pBSNNN was used as a template for amplification. *Taq* DNA polymerase (MBI Fermentas, St. Leon Rot, Germany) was used according to the manufacturer's recommendations. The PCR program used was 5 min at 94°C, followed by 29 cycles of 94°C for 30 s, 60°C (primer pair NPTH/NPTX) or 64°C (DelAH/DelAX; DelBH/DelBX) for 30 s, 72°C for 30 s, and an additional extension step at 72°C for 10 min.

PCR was performed on a T1 Thermocycler (Biometra, Göttingen, Germany) using the specific primers DelAH (5′-GGCGTTAA-CTAGTTGTTATAACTTATAAG-3′), DelAX (5′-CCGCTCGAG-TGAAAGAAGAGG-3′), DelBH (5′-GGCGTTAACCAACCCG-AAATATCG-3′), NOSPH (5′-GGCGTTAACTGAAGGCGGG-3′), and NOSPX (5′-CCGCTCGAGATTATTTGGATTGAG-3′) which harbour either *Hinc*II (primer H) or *Xho*I (X) restriction sites.

Results and discussion

Any promoter driving a selectable marker gene should maintain transcript levels that are sufficient to enable reliable selection of regenerating plants. Consequently, the promoter should be reasonably strong in activity and show a near-constitutive expression pattern, i.e. spatial and temporal regulation should be as broad as possible to ensure that any regenerating tissue type is resistant to the antibiotic.

To identify new promoter sequences that may serve as alternative expression signals in *Physcomitrella* we chose a robust assay that would allow promoter strength to be deduced on the basis of the number of transgenic plants generated after protoplast transformation. A prerequisite for such an approach is a highly standardised transformation protocol, using protoplasts isolated from a very uniformly grown bioreactor culture (Hohe and Reski 2002; Hohe et al. 2002).

For the generation of high numbers of *Physcomitrella* transformants it is necessary to use homologous genomic sequences in the constructs as frequencies of transgene integration increase approximately 10-fold in such cases (Schaefer and Zryd 1997). To enable reproducible and high rates of transgene integration of the different constructs, a well-documented integration platform, the FtsZ1 gene (Strepp et al. 1998), was used. The FstZ1 cDNA served as the backbone for the *nptII* selection cassette, which had been put under the control of different promoters. The three modified FtsZ1 knockout constructs were designed such that the original CaMV 35S promoter fragment was exchanged for one of three other promoters to be tested along with the *npt*II coding sequence followed by the 35S termination signal (Fig. 1). In all four constructs, the FtsZ1 cDNA stretches flanking the selection cassette were 250 and 665 bp in length, respectively. The *npt*II gene confers resistance to the aminoglycosides kanamycin, paromycin, neomycin, and geneticin (G418) (Bevan et al. 1983; Herrera-Estrella et al. 1983). Selection of regenerating plants was performed on G418-containing Knop medium. After transformation and regeneration of protoplasts, the number and phenotype of the resulting plants were compared.

Two sets of independent transformation experiments were conducted and more than 440 stable transgenic plants produced. In the first series, six transformations were performed for each construct and the number of plants that survived the first (S1) and second (S2) round of selection (Fig. 2) were counted. In all experiments, the number of surviving plants was found to be considerably higher after the first selection when compared to numbers

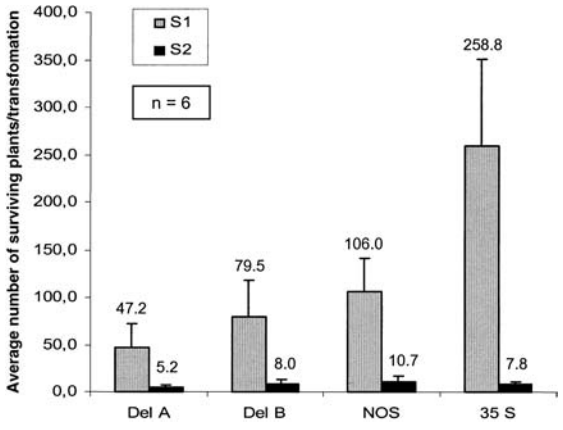

Fig. 2 Graphic representation of the average number of independent transformants surviving the selection process. Resistant plants were generated by transforming protoplasts with linearised DNA of the constructs Lig10/1-DelA (*Del A*), Lig10/1-DelB (*Del B*), Lig10/1-NOS (*NOS*), and Lig10/1 (*35S*). Average numbers of transformants surviving the first (*S1*) and second selection step (*S2*) were compared. Six transformations were performed for each construct. The average numbers of transformants are indicated above each column. *Error bars* show standard deviations

after the second selection. This can be explained by the presence of extra-chromosomal transgenic DNA elements that are expressing the *npt*II gene transiently (Ashton et al. 2000). These elements are lost upon alleviation of selective pressure during the release treatment. Plants surviving the second selection were regarded as stable transgenics, as the rate of confirmed stable transformants is generally very high (98.2%) among *Physcomitrella* plants surviving S2 (Schween et al. 2002).

Interestingly, the 35S-driven cassette resulted in by far the highest number of surviving plants after first selection, contrasting with significantly lower numbers of plants after the second selection. This latter number was comparable among experiments performed with all four constructs. The high numbers of plants generated with the 35S-containing construct could reflect either the higher promoter strength of the 35S promoter in general or an inherent property of the 35S promoter that allows strong transient expression to occur from extra-chromosomal elements under selection pressure. Once integrated into the chromosome, however, all four constructs produced approximately the same number of transgenic plants, arguing in favour of promoter activities that lie in the same range for all four promoters and support sufficiently high NPTII protein levels. The average numbers of plants per transformation surviving the second selection ranged between 5.2±1.9 for construct Lig10/1-DelA, 7.8±3.1 for Lig10/1-35S, 8.0±5.1 for Lig10/1-DelB, and 10.7±6.1 for Lig10/1-NOS. When these numbers are taken as a measure of relative promoter activity, all four promoters are likely to exhibit similar levels of promoter strength in *Physcomitrella* (Fig. 2). These findings were confirmed by an independent replication of these experiments that consisted of four transformations for each construct (data not shown).

Table 2 Activities of the sugar beet V-ATPase subunit c promoter deletion Del A (see Fig. 1) in suspension cells from *Beta vulgaris* and suspension-cultured *P. patens* after transient transformation via particle bombardment. Experiments were performed twice, with ten replicates each. Luciferase activities were determined 20 h after bombardment. Standard errors within one experiment were ≤20%. Promoter activities are calculated as relative light units after calibration against GUS activities from a co-bombarded control plasmid (pFF19G, Lehr et al. 1999). Percent values as compared to 35S in brackets

Organism	Relative light units	
	Del A	35S
B. vulgaris	13,650 (304)	4,490 (100)
P. patens	399 (61)	644 (100)

In both series of experiments, the lowest output of plants was obtained with construct Lig10/1-DelA, followed by Lig10/1-35S and lig10/1Del-B, whereas the highest number of plants was obtained with construct Lig10/1-NOS. These results clearly show that the two BVA/16–1 promoter fragments DelA and DelB can drive *npt*II marker gene expression that allows selection of large numbers of stable moss transformants, comparable to the number obtained with the standard promoters NOS and 35S. The usefulness of the two BVA/16–1 promoter fragments is also supported by the fact that no obvious phenotypical differences were found among BVA/16–1 transformants that could be attributable to weak or aberrant promoter activity.

The method we report here is based on the indirect evaluation of promoter activity. It is not known if stable transgenic plants generated with the four different constructs differ in transgene copy number. It may be possible that the use of weak promoters will result in higher numbers of integrated copies of the corresponding construct. For the moss *Ceratodon purpureus* it has been reported that 35S-*hpt* expression levels were positively correlated with the number of integrated transgene copies (Zeidler et al. 1999). Such differences could compensate for possible differences in promoter strengths between constructs in our present study. To directly address this question, transient LUC reporter gene assays were performed with constructs harbouring the *luc* gene under the control of the 35S and DelA promoters, respectively (Lehr et al. 1999). Bombardment of *Brassica vulgaris* and *Physcomitrella* suspension-cultured cells resulted in DelA rates of gene expression that were in the range of those measured for the 35S promoter. In the case of *Brassica*, the DelA expression rate was 3-fold higher than the 35S promoter activity, which is in line with earlier measurements (Lehr et al. 1999). For *Physcomitrella* the DelA promoter showed a slightly lower rate when compared to the 35S control (Table 2).

The transformation rates (Fig. 2) and the LUC activity measurements (Table 2) in *Physcomitrella* clearly showed that the BVA/16–1 promoter exhibits a strength similar to that of the 35S promoter. Only a few heterologous promoters other than the bacterial NOS and the viral CaMV 35S promoter have been used in *Physcomitrella* (Table 1). In a comparative study utilising the moss *C. purpureus*, the monocot actin promoter Act1 promoted highest and the inducible soybean heat shock promoter very low transgene expression levels (Zeidler et al. 1999).

The sugar beet V-ATPase BVA/16–1 promoter used in this study is the first example of a widely used constitutive promoter from a dicotyledonous plant (Lehr et al. 1999) that has been tested in the moss *P. patens*. As both promoter-fragments gave rise to high numbers of transgenic plants that were comparable to 35S- and NOS-driven constructs, they may also be useful in the production of heterologous proteins in *Physcomitrella*. Moreover, this study reveals that, despite the evolutionary distance between mosses and seed plants, the *Physcomitrella* transcription machinery recognises the whole set of core promoters widely used in genetic transformation of angiosperms.

Acknowledgements We are grateful to Ruth Viereck, University of Heidelberg for providing the V-ATPase BVA/16–1 promoter fragment in the plasmid pBVA/16–1 LUC. The results reported in this manuscript have been obtained in a joint project with BASF Plant Science GmbH.

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