

Cloning and characterization of micro-RNAs from moss

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Summary

Micro-RNAs (miRNAs) are one class of endogenous tiny RNAs that play important regulatory roles in plant development and responses to external stimuli. To date, miRNAs have been cloned from higher plants such as *Arabidopsis*, rice and pumpkin, and there is limited information on their identity in lower plants including Bryophytes. Bryophytes are among the oldest groups of land plants among the earth's flora, and are important for our understanding of the transition to life on land. To identify miRNAs that might have played a role early in land plant evolution, we constructed a library of small RNAs from the juvenile gametophyte (protonema) of the moss *Physcomitrella patens*. Sequence analysis revealed five higher plant miRNA homologues, including three members of the miR319 family, previously shown to be involved in the regulation of leaf morphogenesis, and miR156, which has been suggested to regulate several members of the *SQUAMOSA PROMOTER BINDING-LIKE* (SPL) family in *Arabidopsis*. We have cloned *PpSBP3*, a moss SPL homologue that contains an miR156 complementary site, and demonstrated that its mRNA is cleaved within that site suggesting that it is an miR156 target in moss. Six additional candidate moss miRNAs were identified and shown to be expressed in the gametophyte, some of which were developmentally regulated or upregulated by auxin. Our observations suggest that miRNAs play important regulatory roles in mosses.

Keywords: *Physcomitrella patens*, moss, protonema, small RNA, micro-RNA, auxin.

Introduction

Micro-RNAs (miRNAs) form an abundant class of 21–22 nt non-coding RNAs that are common to diverse species of multicellular life. In higher plants they are major regulators of development (Bartel and Bartel, 2003; Kidner and Martienssen, 2005). miRNAs control gene expression post-transcriptionally by targeting cognate mRNAs for degradation (Llave *et al.*, 2002b; Palatnik *et al.*, 2003; Tang *et al.*, 2003) or by translational repression (Aukerman and Sakai, 2003; Chen, 2004; Reinhart *et al.*, 2000). They are encoded by their own genes as long precursor transcripts ranging from 70 to 300 nt that can form fold-back structures in which the mature miRNAs reside in either the 5' or 3' arm of the hairpin and are processed by *DICER-LIKE1* RNaseIII-like enzyme (DCL1) (Bartel and Bartel, 2003; Baulcombe, 2004). Although miRNA sequences are not conserved between animals and plants, they are highly conserved within kingdoms, and this evolutionary conservation is one of their defining charac-

teristics (Ambros *et al.*, 2003). It has been demonstrated that miRNA 166/165 target sequence is conserved among flowering plants, ferns, lycopods and even mosses, suggesting that miR166/165 is active in lower land plants (Floyd and Bowman, 2004). In addition, in a recent study the expression of several flowering plant miRNAs was detected in a gymnosperm and a fern, and two (miR160 and miR390) were detected in a moss, demonstrating that many plant miRNAs have remained essentially unchanged since before the emergence of flowering plants (Axtell and Bartel, 2005). In addition, miR160 was recently cloned from the moss *Polytrichum juniperinum* (Axtell and Bartel, 2005). However, to date there is very little information on the identity of miRNAs in lower land plants (Kidner and Martienssen, 2005).

Mosses are one of the oldest groups of land plants present among the earth's flora. They originated around 350 Ma, and diverged from flowering plants more than 400 Ma, although

subsequently they have evolved little by comparison with the fossil record (Cove *et al.*, 1997). Insight into the molecular biology of mosses has come mainly from the studies of the model monoecious moss *Physcomitrella patens* (Bryophyta, Musci, Funariaceae) (Cove, 2000), in which efficient homologous recombination allows targeted gene disruption for the study of individual gene function (Schaefer and Zryd, 1997). The moss life cycle is dominated by a haploid gametophyte that is characterized by two distinct developmental stages: the protonema, a filamentous network of chloronemal and caulonemal cells that develop by apical growth and cell division of apical and subapical cells; and the gametophore or leafy shoot, which differentiates by caulinary growth from a simple apical meristem, the bud (Reski, 1998).

In this study we cloned and sequenced a small RNA (sRNA) library from *P. patens* protonema. Our results show that a complex set of sRNAs exists in moss, including at least five higher plant miRNA homologues and several candidate miRNAs unique to moss.

Results

Cloning and sequence analysis of sRNAs from Physcomitrella patens protonemata

The goal of this study was to identify and characterize miRNAs from the moss *P. patens*. We therefore constructed a library of sRNA species from the gametophyte juvenile phase, represented by 7–15-day-old protonemata grown on a minimal medium supplemented with NH_4 -tartrate as a nitrogen source. Under these growth conditions, the protonema is undifferentiated and composed mainly of chloronemal cells (Reski, 1998) (Figure 1a). Small RNAs (15–30 bp) were recovered from approximately 700 μg total protonemal RNA by size fractionation on denaturing polyacrylamide gels, eluted from gel, ligated to 5' and 3' adapters, amplified, restricted by *EcoRI* and *NcoI*, cloned into pGEMT-Easy *EcoRI/NcoI* sites and sequenced. A total of 127 insert-containing clones, randomly selected from the library, were sequenced yielding 234 sRNA sequences, of which 137 were unique and 20 were represented in the library by more than one clone. To identify the putative origin of cloned sequences, a BLASTN search was performed against the *P. patens* public expressed sequence tag (EST) database (<http://moss.nibb.ac.jp/blast/blast.html>) and the National Center for Biotechnology Information (NCBI) non redundant (nr) database. This search revealed that 46 sequences (29.2%) had significant homology to rRNA and tRNA, and 11 sequences (7%) were identical to parts of the *P. patens* chloroplast genome (Sugiura *et al.*, 2003). These sequences were not analysed further. From the remaining 100 sRNA sequences (Table S1), Pp_39 was identical to Arabidopsis MIR319b; Pp_71 was identical to an intergenic region from chromosome 11 of rice; and nine sRNAs corresponded to a

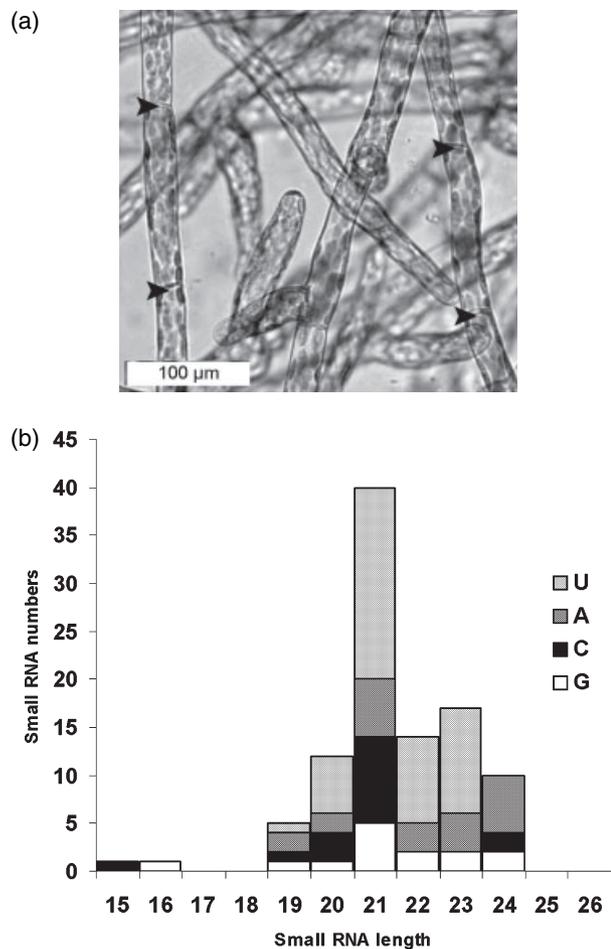


Figure 1. Sequence analysis of *Physcomitrella patens* small RNAs. (a) Bright-field microscopic image of 7-day-old protonema enriched in chloronema that was used for sRNA isolation. Cell boundaries marked by arrows. (b) Size distribution and 5' residue of non-redundant sRNA species contained in our library. RNA fragments that were identified as tRNA, rRNA or chloroplast RNA are not included in this analysis.

moss EST or contig (Table 1). Size distribution of the non-redundant sRNA set ranged from 15 to 24 nt, although the vast majority contained 20–24 nt, with the 21 nt size most abundant (Figure 1b). The majority of the 20–23 nt sequences (Figure 1b) had a uridine at the 5' terminus, as in most miRNAs from higher plants (Bartel and Bartel, 2003).

BLASTN search against the miRNA Registry (Ambros *et al.*, 2003) and the *Arabidopsis thaliana* Small RNA Project database (<http://asrp.cgrb.oregonstate.edu>) identified five miRNA homologues within our non-redundant sRNA set (Table S1). Pp_7 and Pp_73 appeared twice each and were identical to Arabidopsis miR390 and miR156, respectively. A group of three related sRNAs: Pp_38 (Pp-miR319a), Pp_39 (Pp-miR319b) and Pp_37 (Pp-miR319c), were almost identical to miR319b except that they had an extra cytosine at their 5' end (Figure 2a). We noticed that the Arabidopsis and rice

Table 1 *Physcomitrella patens* sRNAs identical to an EST contig

Small RNA				<i>P. patens</i> contig/EST ^a		
ID	Sequence	Length (nt)	Orientation	ID	BLASTX best hit ^b	Best hit annotation
Pp_5	AAGAGGAUAAAAGUGUGUCCUAGGG	24	Antisense	Contig12715	NS	–
Pp_17	AGGCAGUAGAAGAGCACCGGA	21	Antisense	Bl488366	NS	–
Pp_19	AUAUACAACCGAUACAGGAG	20	Antisense	Bl488366	NS	–
Pp_38	CUUGGACUGAAGGGAGCUCCC	21	Sense	Contig3792	NS	–
Pp_40	GACACGCAGACACGCGCGGCC	21	Antisense	Bl488366	NS	–
Pp_57 ^c	UAGUUACCUCGAAUUAUUGGGAGC	23	Sense	Contig621	CAB39733	<i>Drosophila</i> retrotransposon Osvaldo
Pp_60	UAUGUCCAUUGCAGUUGCAUAC	22	Sense	Contig10605	NS	–
Pp_61	UCAAGGGAUUGAAGGCAUGG	20	Sense	Contig12207	CAD22096	<i>P. patens</i> adenosine 5' phosphosulfate reductase
Pp_95	UUGCAUGGAGUCUAUGUCUGGA	22	Sense	AW155950	NS	–

NS, not significant, *E*-value >0.001.

^aBLASTN search done against the *P. patens* public EST database.

^bBLASTX search done against the GeneBank and TAIR databases.

^cIdentical to an EST except for the first nucleotide.

miR-319b precursors are 100% identical to Pp-miR319b, including an extra cytosine at the 5' end of the mature miR319b sequence. This raises the possibility that a longer miR319 with 5' cytosine may be processed from these precursors, although only the short version has been cloned to date. It also suggests a possible evolutionary relationship between Pp-miR319b and higher plant miR319b. It is noteworthy that Pp-miR319b was the most abundant sRNA in our library, appearing 24 times (10.3%). Sequence alignment revealed four additional groups of sRNAs in our library: the Pp_89 group, which had seven members and a length of 20–23 nt (Figure 2b); and the Pp_27 (Figure 2c); Pp_60 (Figure 2d); and Pp_94 (Figure 2e) groups with two members each. Comparison of our sRNA set with the recently cloned sRNA set from the moss *P. juniperinum* (Axtell and Bartel, 2005) revealed only four identical sequences: Pp_13, Pp_31, Pp_89 and Pp_95.

To find out whether there were any moss-specific miRNAs in our database, we searched for sequences that, like flowering plant miRNAs, could have been processed from the transcribed (sense) strand of a capped and polyadenylated pri-miRNA. Such pri-miRNAs would appear as non-coding ESTs in which the miRNA region would correspond to a partially double-stranded region (Kurihara and Watanabe, 2004). From the nine sRNA sequences that correspond to a moss EST or contig, six, including the Pp-miR319a, were each 100% identical to an EST contig in the sense polarity (Table 1). BLASTX analysis suggested that three of these ESTs [contig 10605 (Pp_60); AW155950 (Pp_95); contig 3972 (Pp-miR319a)], which are predicted to encode no or short open reading frames (ORFs) of unknown function, might represent non-coding pri-miRNA.

Once transcribed, plant pri-miRNAs are processed into miRNA precursor transcripts ranging from 70 to 300 nt with stable hairpin structures in which the mature miRNAs reside

in either the 5' or 3' arm (Bartel and Bartel, 2003; Kurihara and Watanabe, 2004). Therefore each EST contig sequence surrounding Pp_60, Pp_95 and Pp-miR319a was analysed for its ability to fold into hairpin structures using the MFOLD program (Zuker, 2003). The stability of the resulting secondary structure was tested statistically using the RANDFOLD program (Bonnet *et al.*, 2004). This analysis suggested that sequences of 170, 202 and 120 nt of contig 3972 (Pp-miR319a), contig 10605 (Pp_60) and EST AW155950 (Pp_95), respectively, can form stable (RANDFOLD = 0.001) fold-back structures, and contain the corresponding sRNA within one arm of the hairpin (Figure 3a), indicating that these sequences are putative miRNA precursors. To test whether Pp_71, which perfectly matched a rice intergenic sequence on chromosome 11 (AC136786:116680-116660), is a putative miRNA, a similar folding analysis was also carried out on 300 bp of AC136786 spanning Pp_71. This analysis indicated that 94 nt of that sequence can form a stable (RANDFOLD = 0.001) fold-back structure containing Pp_71 within one arm of the hairpin (Figure 3b), suggesting that it represents a putative rice miRNA precursor and that Pp_71 might represent a yet unidentified candidate miRNA in rice. Together our analyses suggest that pri- and pre-miRNAs in moss are similar to those in higher plants, and that Pp_60, Pp_71 and Pp_95 are *P. patens* candidate miRNAs.

Other sRNAs did not have the characteristics of miRNAs, but instead resembled endogenous siRNAs found in higher plants and other organisms. One sRNA (Pp_57) was identical in the sense polarity to contig 621 with one mismatch. Contig 621 encodes an ORF that has short but significant similarity (*E* value of 1E-06) to a retrotransposon polyprotein from *Drosophila*, soybean, maize and rice, which might suggest that Pp_57 represents a transposon-related sRNA. In higher plants, several sRNAs that correspond to transposons have been cloned (Llave *et al.*, 2002a; Yoo *et al.*, 2004).

(a) Pp-miR319		
At-miR319c	-UUGGACUGAAGGGAGCUCCU	
At-miR319b	-UUGGACUGAAGGGAGCUCCC	
Pp-miR319a	CUUGGACUGAAGGGAGCUCC-	2
Pp-miR319b	CUUGGACUGAAGGGAGCUCCC	24
Pp-miR319c	CUUGGACUGAAGGGAGCGCC-	1
(b) Pp_89		
Pp_70	-UCGUGCCAAG-CUGUGUGCAAC	5
Pp_90	UUCGUGCCAAG-CU-UGUGCAA-	1
Pp_91	UUCGUGCCAAG-uUGUGUGCAAC	1
Pp_85	UUCGUGCCAaACUGUGUGCAA-	1
Pp_86	UUCGUGCCAaACUGUGUGCAu-	1
Pp_87	UUCGUGCCAAGACUGUGUGCAAC	1
Pp_89	UUCGUGCCAAG-CUGUGUGCAAC	9
(c) Pp_27		
Pp_27	ccCUCACAGcCUGccCAGCUC--	1
Pp_34	--CUCACAGuCUGcaCAGCUCuc	1
(d) Pp_60		
Pp_60	UAUGUCCAUUG-CAGUUGCAUAC	5
Pp_59	UAUGUCCAUUGaCAGUUGCAUAC	1
(e) Pp_94		
Pp_92	UUGAGaUGUUUCUACAGGCUA	1
Pp_94	UUGAGgUGUUUCUACAGGCUA	9

Figure 2. *Physcomitrella patens* groups of sequence-related small RNAs. (a–e) Sequences were aligned with the CLUSTALW program (Thompson *et al.*, 1994). Identical and conserved nucleotides are labelled in upper-case letters; lower case represent changes in nucleotide sequences in some group members. Number of times each sequence is represented in our library is shown on the right. Sequences appearing once were verified by sequencing of both strands. Pp, *P. patens*; At, *A. thaliana*.

Four sRNAs corresponded to EST contigs in the antisense polarity (Table 1). Interestingly, three of these, Pp_17 (25–5), Pp_40 (69–49) and Pp_19 (88–69), corresponded to different regions of the antisense strand of EST BI488366. BI488366 is a 250 bp cDNA isolated from 7-day-old, auxin-treated protonema, which contains a 48 amino acid partial ORF with no significant similarity, starting from its 5' end. These three sRNAs resemble the *trans*-acting siRNAs found in Arabidopsis (Allen *et al.*, 2005; Peragine *et al.*, 2004; Vazquez *et al.*, 2004) in that they appear to be at least partially in phase with each other: there is a one nucleotide overlap between Pp_40 and Pp_19, and 23 nt difference between Pp_40 and Pp_17. The presence of miR390 in *P. patens* is consistent with that suggestion, as a *trans*-acting, siRNA-generating transcript (TAS3) was identified as target of miR390 in Arabidopsis (Allen *et al.*, 2005). However, a significant homology was not found between BI488366 and TAS3 (not shown). At present we have not characterized the biogenesis of Pp_17, Pp_19 and Pp_40 but, as they are derived from the antisense strand of an EST, it is likely (as with *trans*-acting siRNAs) that an RNA-dependent RNA polymerase would be involved.

The rest of the cloned sRNAs (Table S1) did not correspond to any publicly available moss EST or genomic sequence. Moreover, BLASTN analysis against The Arabidopsis Information Resource (TAIR) and the NCBI nr database suggested that these sRNAs did not have significant similarity to an EST or intergenic sequence from Arabidopsis or any other higher plant.

Expression analysis of cloned miRNAs and candidate miRNAs

As genomic sequence information for *P. patens* is currently unavailable, and because the public EST database is incomplete, sequence analysis of cloned sRNAs identified a limited number of miRNAs. Nevertheless, it has been reported that multiple clones have been found in Arabidopsis sRNA libraries for nearly all reported miRNAs, suggesting that they are more abundant than each of the non-miRNA species, most of which have been found only once (Bartel and Bartel, 2003; Sunkar and Zhu, 2004). Therefore, in order to identify additional candidate miRNAs, we analysed the expression of eight *P. patens* sRNAs that appeared more than once in our library (Pp_13, Pp_17, Pp_42, Pp_43, Pp_75, Pp_82, Pp_89, Pp_94; Table S1). Similar expression analysis was also performed for miRNA homologues (Pp-miR319b, miR156, miR390) and for strong candidate miRNAs (Pp_60, Pp_71, Pp_95) to assist in the determination of their function in moss. Expression analysis was performed by RNA gel blot of total RNA from isolated 7-day-old protonema and 30-day-old gametophore representing juvenile and adult gametophyte, respectively. In addition, expression was analysed in whole-moss culture grown on a minimal medium every week for 4 weeks of development, starting from subcultured protonema. As shown in Figure 4(a), we were able to detect a positive signal of the expected size, confirming the expression of miRNA homologues Pp-miR319b, miR390 and miR156; of strong candidate miRNAs Pp_60, Pp_71 and Pp_95; and of sRNAs Pp_42, Pp_89, and Pp_94, which were each relatively abundant and represented by at least five sequences in our library (Table S1). No hybridization was detected with end-labelled sense oligonucleotides as probes for sRNAs Pp_42, Pp_60, Pp_71, Pp_89, Pp_94 and Pp_95 (not shown).

Pp-miR319b, miR390, miR156, Pp_89, Pp_95 and Pp_71 were detected at similar levels in both juvenile and adult gametophyte (Figure 4a). Nevertheless, Pp_60, Pp_94, and Pp_42 showed differential expression. Pp_60 was detected mainly in 1-week-old undifferentiated protonema, but was almost absent from gametophores (Figure 4a). Furthermore, the levels of Pp_60 dropped after 2 weeks' protonemal growth (Figure 4a) when only few young gametophores were visible in culture, indicating that Pp_60 is specific to non-differentiating protonema composed mainly from chloronema cells. In contrast to Pp_60, Pp_42 and Pp_94 were

(a)

Contig 3792: $\Delta G = -99$, 0.001

```

          G          U  UG   CGA      -  U  CGCUG  AAU  C   C   U  U          GC
418-5' GUGGAGCUCU UUUCGGUCCAA AG  GCUG  CGGAAGGU GG CC      CCG  CA ACGU CGGGU CC UAUCGGGG A
587-3' CACCUCGAGG GAAGUCAGGUU UC  CGGC  GCCUUCCA CU GG      GGC  GU UGCA GCCUA GG AUAGCCCC G
          -          C  UG   CC-      G  -  UCAAG  CCU  U   A   U  C          GG

```

Contig 10605: $\Delta G = -75$, 0.001

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105-5' AA|   C          UGU      G   --   U  UG   A-   .-AA   A  U  U   ---  GA   UU
        AUAUG AUGCAACU  GUGGACA ACUGA  CUAGUC AG  GUGU  GUGG   AGUAUU GU GGU GAUG  CAC  UGUUG \
        UAUAC  UACGUUGA  UACCUGU  UGACU  GAUCAG UC  UACA  CACC   UCAUAA CG UCG CUGC  GUG  ACAAC  U
306-3' --^   A          CGU      A   UA   C  GU   CC (35nt side loop) - - -   UCA  A-   GU

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AW155950: $\Delta G = -49$, 0.001

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139-5'          U   G  U          -   UU   -----  UCCCG  AA  U
        CUCUAACAU GCAUG AG CUAUGUCU GGAGUA GUCCU   UCAU   UGCA  UGC G
        GAGAUUGUA CGUAU UC GAUGCAGA CCUUAU  CAGGG   AGUG   ACGU  AUG A
259-3'          -   -  C          A   --   UUACA  UAAGA  A-  A

```

(b)

AC136786: $\Delta G = -68.3$, 0.001

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116685-5'          A          C          -----  UCA  U
        GGCGGUGACAACG GAGAGACACGC GGUGCG      GCGG  CGG G
        CCGUCACUGUUGC CUCUUUCGUGCG CCAUGC      CGCC  GCC A
116592-3'          C          U          GGCGG  CG-  G

```

Figure 3. Predicted fold-back structures of putative miRNA precursors.

(a, b) Positions of nucleotides used in each EST or genomic sequence are indicated. Free energy (ΔG) and randomization test scores as calculated by RANDFOLD (Bonnet *et al.*, 2004) are shown. Pp-miR319a (contig 3972), Pp_60 (contig 10605), Pp_95 (AW155950) and Pp_71 (AC136786) sequences are underlined. The actual size of each putative precursor was not identified experimentally and may be slightly shorter or longer than represented.

expressed mainly in mature gametophores and were much less abundant in non-differentiated 7-day-old protonema (Figure 4a). Consistent with this observation, Pp_42 and Pp_94 abundance increased with time as more adult gametophores were visible in culture, with maximal levels at 28-day-old culture (Figure 4a).

To test if the unique *P. patens* strong candidate miRNAs and sRNAs are conserved in other mosses, RNA gel-blot analysis of total RNA extracted from naturally grown *Funaria hygrometrica* gametophores was performed. We were able to detect a positive signal of the expected size, confirming the presence of sRNA Pp_42, Pp_71, Pp_89, Pp_94 and Pp_95 homologues in *F. hygrometrica* (Figure 4b). A positive signal was not detected for the chloronema-specific Pp_60, but this could result from the fact that Pp_60 is not expressed in *Funaria* gametophores as in *P. patens*.

Long- and short-term effects of exogenous NAA on differentially expressed candidate miRNAs

Auxin induces the first differentiation step of the *P. patens* protonema: the transition from chloronema to caulonema

(Ashton *et al.*, 1979; Johri and Desai, 1973). It has been shown that the production of caulonemata is specifically increased by application of 2.5–50 μM 1-naphthalene acetic acid (NAA) (Ashton *et al.*, 1979; Johri and Desai, 1973). We analysed the effect of increased caulonema/cloronema proportions on the levels of differentially expressed sRNAs Pp_42, Pp_60, and Pp_94 by RNA gel blot of total RNA extracted from 1-week-old subcultured protonemata grown in the presence or absence of 5 μM exogenous NAA. As a control, the expression level of constitutive Pp_71 was also analysed. As shown in Figure 5(a), Pp_42 was strongly affected by 1 week's NAA treatment, and levels increased 10-fold (Figure 5a). Levels of the gametophore-abundant Pp_94 also increased, but only by 50%. In contrast, the levels of the chloronema-specific Pp_60 decreased in NAA-treated protonema, which contained a higher caulonema/cloronema ratio (Figure 5a). In contrast, the levels of Pp_71 were not markedly changed on 1 week's exposure to NAA. These results prompted us to test whether Pp_60, Pp_94 and Pp_42 are directly regulated by NAA. Their levels were examined by RNA gel-blot analysis of 1-week-old protonema treated or not treated with 5 μM NAA for a period of 6 or 24 h.

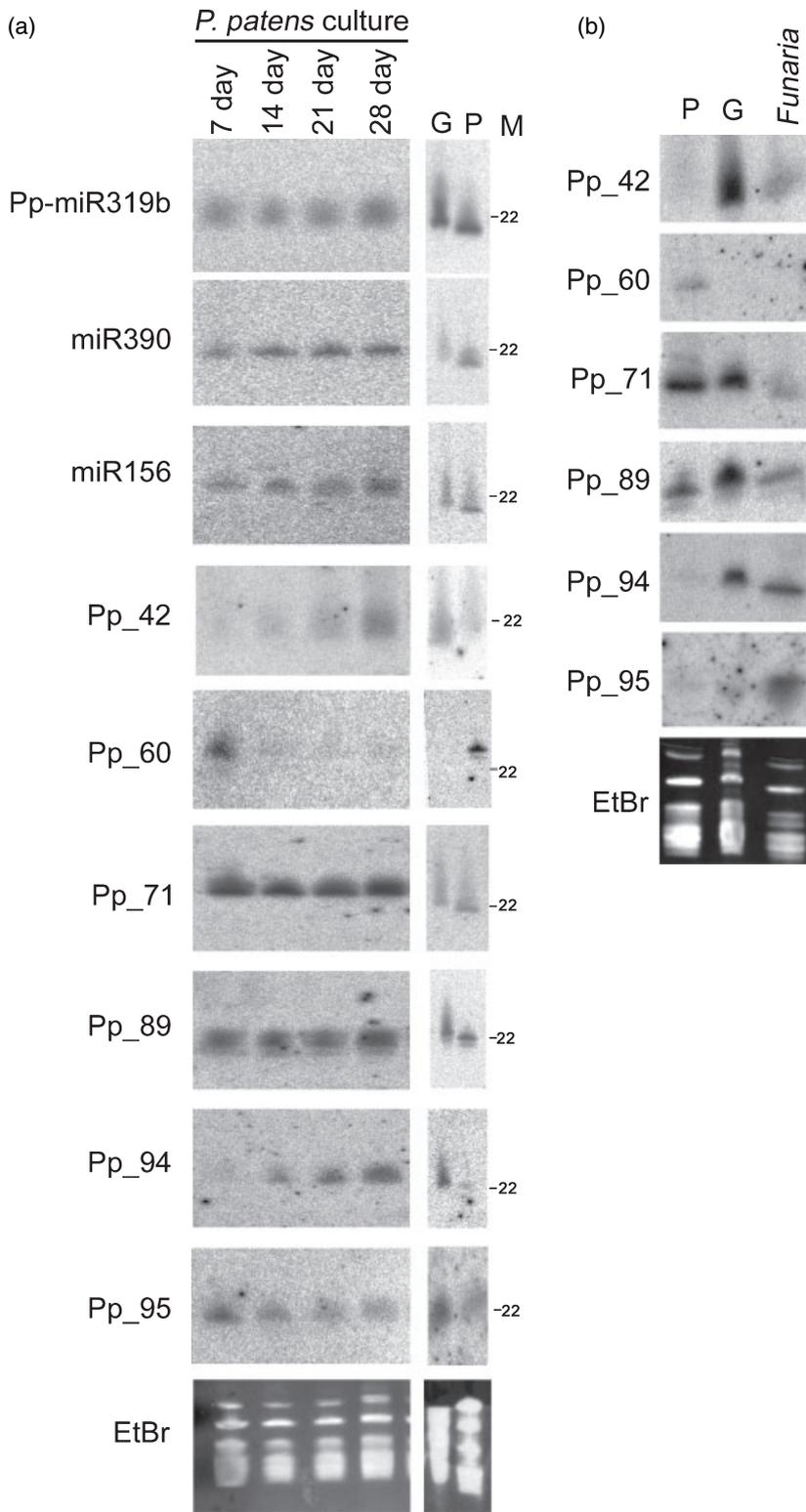


Figure 4. RNA gel-blot analysis of miRNAs and smRNAs in *Physcomitrella patens* and *Funaria hygrometrica* gametophyte.

Total RNA was extracted from isolated 7-day-old *P. patens* protonema (P); isolated 30-day-old *P. patens* gametophores (G); and either (a) *P. patens* culture at indicated days post-inoculation of homogenized protonema on a minimal medium, or (b) naturally grown *F. hygrometrica* gametophores. RNA samples (30 µg) were separated on a denaturing polyacrylamide gel, blotted and probed with oligonucleotide probes complementary to the indicated sRNA sequence. Each sample was harvested from at least five independent Petri dishes, except for *F. hygrometrica* gametophores which were picked from nature. The blots were stripped and re-probed with the indicated sRNAs. The tRNA and 5S rRNA bands were visualized by ethidium bromide staining of polyacrylamide gels and served as loading controls. Nucleotide size marker, M.

This treatment did not result in any observable developmental changes in the protonema (not shown). However, following 6 h exposure to 5 µM NAA, a 1.7-fold increase in the level of Pp_94 was observed, and a 2.4-fold

increase after 24 h exposure to NAA (Figure 5b). No notable changes were observed in the levels of Pp_42, Pp_60, and the constitutively expressed Pp_71 (Figure 5b).

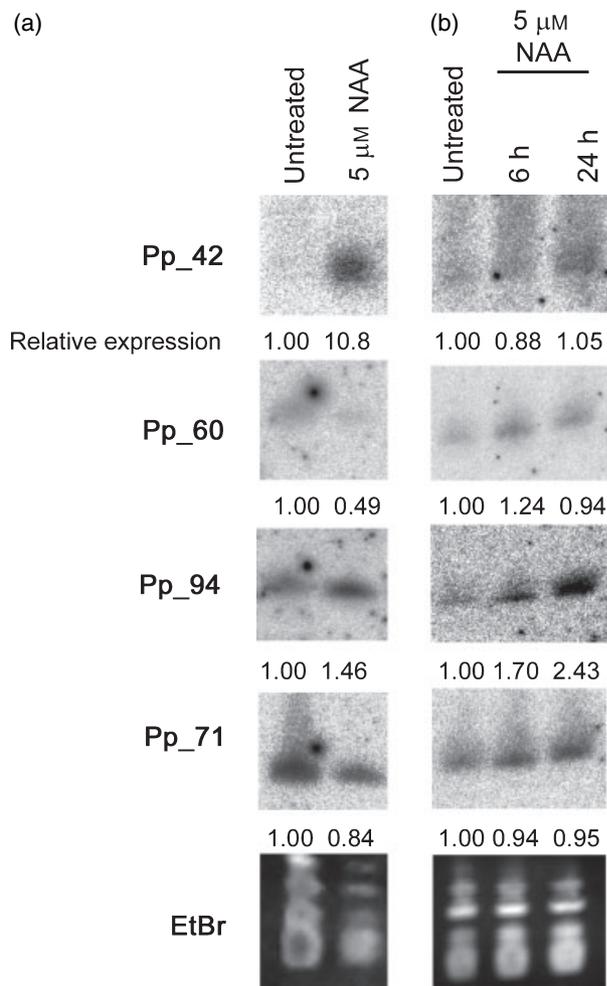


Figure 5. Long- and short-term effects of 1-naphthalene acetic acid (NAA) on the levels of differentially expressed Pp_42, Pp_60, Pp_94 and constitutively expressed Pp_71.

RNA gel blots of (a) 30; or (b) 45 μg total RNA prepared from protonema grown for (a) 7 days on a minimal NH₄ medium supplemented with 5 μM exogenous NAA; or (b) exposed to 5 μM NAA for 6 or 24 h. Each sample was harvested from at least five independent Petri dishes. Blots were probed with oligonucleotides probes complementary to the indicated sRNA sequence. Blots were stripped and re-probed with the indicated sRNAs. The tRNA and 5S rRNA bands were visualized by ethidium bromide staining of polyacrylamide gels and served as loading controls. Expression levels relative to untreated tissue were determined after normalization based on ethidium bromide staining and are indicated below each panel.

Predicted and validated targets of cloned and candidate miRNAs

To identify potential miRNA regulatory targets, we searched the *P. patens* public EST database for mRNAs that were complementary, with three or fewer mismatches (Rhoades *et al.*, 2002), to at least one of the miRNAs or candidate miRNAs expressed in moss (Figure 4a) or their group members (Figure 2). Gaps were not allowed, and the G:U non-canonical pair was treated as a mismatch. Only five potential regulatory

targets with three mismatches were found (Table 2). These targets were in accordance with the empirical parameters for target recognition as formulated by Schwab *et al.* (2005). Contig3294, which corresponds to Pp_90, is predicted to encode a Petunia Nam and Arabidopsis ATAF1, ATAF2 and CUC2 (NAC) domain containing protein that has a closely related homologue in Arabidopsis. NAC-domain genes were previously demonstrated to be regulated by miR164 in Arabidopsis (Mallory *et al.*, 2004). Contig 7486, which corresponds to Pp_71, is predicted to encode a protein highly similar to Arabidopsis brassinosteroid positive regulator-related protein. The Pp_71 interaction site lies upstream of the ORF, probably in the 5' UTR. The other potential targets were predicted to encode proteins of unknown function.

Of the 16 SQUAMOSA promoter binding-like (SPL) genes in Arabidopsis (Riechmann *et al.*, 2000), 10 are predicted to have miR156 complementary sites (Rhoades *et al.*, 2002). SPL2 (Boutet *et al.*, 2003; Kasschau *et al.*, 2003) and SPL3 (Chen *et al.*, 2004) cleavage products, consistent with cleavage being guided by members of the miR156 family, were previously identified in Arabidopsis. We identified a moss EST sequence containing an SBP-box and used it as a probe to screen a *P. patens* cDNA library, resulting in the subcloning of a full-length cDNA designated *PpSBP3*. Sequence analysis revealed that the *PpSBP3* gene had a conserved SBP box and a downstream sequence of near-perfect complementarity to miR156, indicating that it may serve as its target (Figure 6). To test this possibility, target cleavage analysis of *PpSBP3*, using RNA ligase-mediated 5' RACE as described by (Llave *et al.*, 2002b), was performed on mRNA isolated from 1-week-old protonema. A single PCR product of the size predicted to be generated from cleaved *PpSBP3* template could be amplified (Figure 6a). This product was cloned, and the sequence of 12 independent inserts was determined. At least 83% of the cloned product 5' ends terminated at a position corresponding to the middle of the region of complementarity with miR156 (Figure 6b), suggesting that it represents a *bona fide* miRNA-mediated *PpSBP3* cleavage product.

Discussion

Within the land plant lineage, bryophytes diverged at the most basal position. Fossil records suggest that mosses have undergone only small morphological changes since then (Cove *et al.*, 1997; Miller, 1984), and thus can be considered as a 'snapshot' of an early moment in land plant evolution. A previous study has demonstrated that miR166/165 target sequence is conserved in class III HD-ZIP gene homologue from *P. patens*, suggesting that miRNA 165/166 functions in moss (Floyd and Bowman, 2004). Moreover, miR160 was recently cloned from the moss *P. juniperinum* (Axtell and Bartel, 2005). By sequencing a library of sRNAs from the moss *P. patens*, we are able to provide evidence for

Table 2 Prediction of *Physcomitrella patens* miRNA and candidate miRNA targets

Clone ID	Predicted target contig ^a	Contig/EST coordinates	BLASTX best hit ^b	Best hit annotation	E-value
Pp_89	Contig6575 (3/0)	379–358	At2g42040	Unknown	0.005
Pp_90	Contig 2820 (3/0)	95–76	–	Unknown	–
Pp_90	Contig3294 (3/1)	343–324	NP_187845	NAC-domain family	4E-46
Pp_71	Contig7486 (3/0)	976–956	NP_565187	Brassinosteroid signalling positive regulator-related	5E-26
miR156	Contig 2071 (3/0)	843–824	–	Unknown	–

^aIn parentheses, total number of mismatches/number of G:U wobbles.

^bBLASTX search done against the GeneBank and TAIR databases.

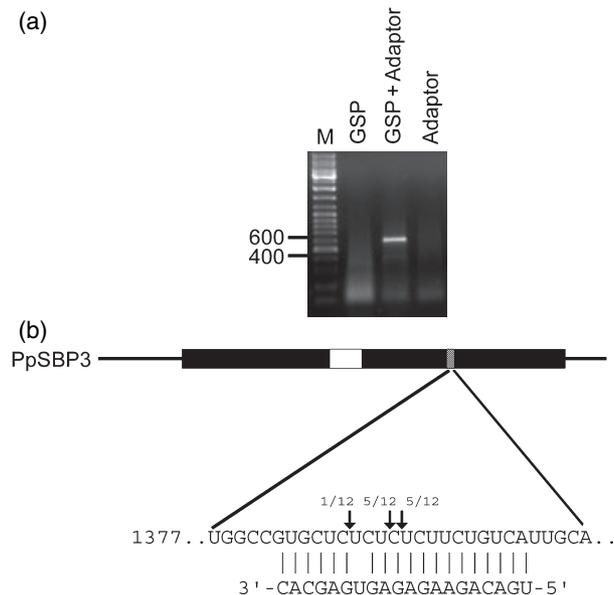


Figure 6. Determination of PpSBP3 cleavage site by RNA ligase-mediated 5' RACE.

(a) Ethidium bromide-stained agarose gel showing the 5' RACE product. The GeneRacer 5' primer (Adaptor) and PpSBP3 3' primer (GSP) used for each RACE reaction are indicated above. Lane M, 100 bp DNA ladder.

(b) Schematic representation of PpSBP3 mRNA cleavage sites. Black box represents an ORF; flanking thin lines represent non-translated regions. The putative SBP-box and miRNA interaction site are shown by white and grey boxes, respectively. The miRNA sequence and partial sequence of PpSBP3 mRNA are shown in the expanded region. Watson-Crick pairing (vertical dashes) is shown. Arrows indicate the positions of inferred cleavage sites. The number of the 5' RACE clones that correspond to each site is indicated.

the existence of at least three additional groups of higher plant miRNAs (miR319, miR156, miR390) very early during land plant evolution. Moreover, Arabidopsis and rice miRNA homologues are always encoded by the same arm of their precursor hairpin (Reinhart *et al.*, 2002). We have identified a putative Pp-miR319a precursor and found that, like the Arabidopsis and rice miR319 (Palatnik *et al.*, 2003), the miRNA is encoded in the 3' arm of the hairpin, extending the conservation between flowering plants and mosses to the MIR gene structure. RNA gel-blot analysis of *P. patens* total RNA with oligonucleotide probes against 12 other

conserved higher plant miRNAs (excluding miR160 and miR171), as listed by Bartel and Bartel (2003) – including miR157 and miR159, close relatives of miR156 and miR319, respectively – failed to give a positive signal (our unpublished results). One possible explanation is that miR156, miR160, miR166, miR319 and miR390 are members of a limited group of 'ancient' miRNAs that have played conserved regulatory roles since the last common ancestor of mosses and seed plants. Indeed, a cleavage product of moss mRNA homologues consistent with cleavage being guided by miR165/166 (Floyd and Bowman, 2004) has been identified: miR160 (Axtell and Bartel, 2005). An mRNA product consistent with miR156-mediated cleavage has been detected by us for *PpSBP3*, a moss SPL homologue.

In Arabidopsis, miR319 can guide the mRNA cleavage of several *TCP* genes (Palatnik *et al.*, 2003), which represent Arabidopsis homologues of the snapdragon *CINCINNATA* gene, involved in the control of leaf development and morphology (Nath *et al.*, 2003). Both the *MIR-319* precursor and miR-319 target motif in *TCP* genes are found in a wide range of flowering plants, suggesting a conserved role in controlling leaf morphogenesis (Palatnik *et al.*, 2003). We have found that Pp-miR319b is highly abundant in the leafy gametophore, raising the possibility that members of Pp-miR319 family may play a role in gametophore leaf morphogenesis. This possibility is intriguing as mosses display the beginnings of differentiation into stems and leaves (Cove *et al.*, 1997), and because gametophore leaf structures, which are very simple and usually composed of only one layer of cells (Reski, 1998; Schaefer and Zryd, 2001), may be a very attractive system for studying how a flat sheet of cells acquires its shape.

Two criteria are generally accepted to distinguish miRNAs from other classes of sRNAs: the ability of the RNA surrounding the mature miRNA sequence to fold into a hairpin miRNA precursor; and the phylogenetic conservation of miRNAs (Ambros *et al.*, 2003). Therefore, in the absence of a full genomic sequence of *P. patens* and a complete EST database, defining unique moss miRNAs may be problematic. Nevertheless, we have identified in the moss EST database putative miRNA-like hairpin precursors for Pp_60 and Pp_95. In addition, we have identified in rice a

putative miRNA hairpin precursor, which is predicted to give rise to rice Pp_71 miRNA homologue, suggesting that Pp_71 (like Pp_60 and Pp_95) represents strong candidate miRNA. Pp_60 (5/234), Pp_71 (5/234) and Pp_95 (9/234) were relatively abundant sequences in our library, and this is consistent with reports in Arabidopsis sRNA libraries that nearly all miRNAs have been found multiple times and are more abundant than each of the non-miRNA species, most of which have been found only once (Bartel and Bartel, 2003; Sunkar and Zhu, 2004). Similarly, the expression of abundant Pp_42, Pp_89 and Pp_94, which appeared at least five times each in our library (Table S1), has been detected. In addition, we have also detected the presence of homologues of these sRNAs in the gametophores of *F. hygrometrica*, a related moss species that belongs (like *P. patens*) to the *Funariaceae*. Moreover, Pp_89 and Pp_95 homologues were recently cloned from the moss *P. juniperinum* which belongs to the *Polytrichaceae* (Axtell and Bartel, 2005). Thus it is highly likely that Pp_42, Pp_89 and Pp_94 represent candidate miRNAs which have been conserved in mosses. However, because their miRNA-like hairpin precursors could not be identified, we cannot rule out the possibility that each might represent another class of endogenous sRNA.

The tissue- and development-specific expression of miRNAs may provide clues about their physiological function. Many known higher plant miRNAs are differentially expressed during development (Reinhart *et al.*, 2002; Sunkar and Zhu, 2004; Wang *et al.*, 2004). In moss we have observed several distinct sRNA expression patterns in the gametophyte. miR156, Pp-miR319b, miR390, Pp_71 and Pp_89 were expressed at similar levels in the juvenile and adult phases of the gametophyte. Pp_94 and Pp_42, on the other hand, were highly expressed in the gametophore but much less abundant in the juvenile gametophyte or protonema, suggesting that, unlike the constitutively expressed miRNAs, they function mainly in the adult gametophyte stage. Nevertheless, growing protonema in the presence of exogenous NAA, which is known to enhance the production of caulonema cells (Ashton *et al.*, 1979; Johri and Desai, 1973), increased Pp_42 and, to a lesser extent, Pp_94 expression levels, indicating that they may also be abundant in differentiating caulonema cells. The transition from chloronema to caulonema is known to correspond to a growth period of about 24 h (Reski, 1998), therefore the specific upregulation of Pp_94 after 6 h NAA treatment might suggest that it is directly regulated by auxin (Figure 5b). This upregulation might be needed in order to clear transcripts, which maintain the non-differentiated state of the protonema and may be one of many cellular events that are regulated by auxin and lead to chloronema differentiation. In Arabidopsis, miR164 was recently demonstrated to be induced by auxin (Guo *et al.*, 2005). In contrast to Pp_94 and Pp_42, Pp_60 was detected almost entirely in non-differentiated protonema that is mainly composed from chloronema cells (Figure 4a).

Consistent with that, its levels decreased on long NAA treatment which converted chloronema to caulonema, but did not change significantly following very short NAA treatment (Figure 5b). Based on this result, we speculate that Pp_60 might function in an opposite way to Pp_94 and downregulate one of the factors that regulates chloronema differentiation.

A diverse population of sRNAs in *P. patens* was identified in this study. These sRNAs are clearly related to higher plant sRNAs based on size (predominantly 20–24 nt in length) and inferred terminal structure, which also suggest that they are formed by an RNaseIII-like mechanism. As in higher plants (Baulcombe, 2004), we can identify in this population miRNAs, putative transposon siRNAs and endogenous sRNAs reminiscent of *trans*-acting siRNAs. However, their size distribution profile (Figure 1b) is somewhat different from that of Arabidopsis sRNAs (Llave *et al.*, 2002a), which contain a high proportion of 24 nt molecules. One possibility is that there are differences between moss and higher plant DCL genes. Indeed, different Arabidopsis DCL proteins have been implicated in the generation of distinct sRNA size classes (Xie *et al.*, 2004). At present, querying of the current *P. patens* EST database with various Arabidopsis DCL-coding sequences (Schauer *et al.*, 2002) does not reveal a significant match to an RNaseIII domain. Finally, the sRNA and miRNA sequences identified in this study are expected to provide interesting insights on moss miRNA regulation and sRNA silencing mechanisms, which played a role soon after plants inhabited the land.

Experimental procedures

Plant material and culture conditions

Protonemata of *Physcomitrella patens* ssp. *patens* (Ashton and Cove, 1977) were cultured on a solid minimal medium described by Ashton *et al.* (1979) supplemented with 2.7 mM NH₄ tartrate. Cultures were grown in 9 cm Petri dishes on medium solidified with 0.7% agar (Merck 1614) and overlaid with 8 cm diameter cellophane disc (type 325P; AA Packaging Ltd, Preston, UK). Plates were grown in a culture room at 24 ± 1°C. Light was provided from above by two to four fluorescent tubes (Osram L 18W/10, Munich, Germany) under a regime of 16 h light and 8 h darkness. Moss protonema was subcultured every 7 days using a Polytron homogenizer (type PT 1600E; Kinematika, Lucerne, Switzerland). For adult gametophore isolation, small pieces of 1-week-old healthy protonemal tissue were inoculated for 30 days onto solid minimal medium in 90 mm diameter Petri dishes, under the above temperature and light conditions. Adult gametophores were then isolated by cutting their stem a few millimetres above the agar with fine scissors. To examine the long-term effects of exogenous auxin and cytokinin on sRNA expression, homogenized protonema was subcultured for 1 week on minimal NH₄ medium covered with cellophane and supplemented with 5 µM 1-naphthalene acetic acid (NAA, Sigma, Rehovot, Israel). To examine the short-term effects of exogenous NAA, cellophane discs with 1-week-old protonema were transferred to Petri dishes with a minimal medium supplemented with 5 µM hormone.

Nucleic acid isolation

Total RNA was extracted from protonemata or gametophores using TRI reagent (Sigma) according to the manufacturer's protocol, except that the upper phase containing RNA was re-extracted twice to three times more with 25:24:1 phenol/chloroform/isoamyl alcohol to remove sRNA-binding proteins. After addition of isopropanol, the RNA extract was incubated overnight at -20°C (instead of 5 min at room temperature) to enhance precipitation of low-molecular-weight RNA. Following an ethanol wash, RNA was resuspended in 50% formamide (Sigma) and kept at -80°C until use.

Small RNA cloning

sRNA cloning was performed essentially as described by Llave *et al.* (2002a). Briefly, total RNA (750 μg) was resolved by electrophoresis on denaturing 15% polyacrylamide gel. A gel slice containing RNAs of approximately 15–35 nt (based on a DNA oligonucleotide size standard) was excised and eluted in 0.3 M NaCl at 4°C for approximately 16 h. Eluted RNAs (smRNAs) were precipitated in ethanol, resuspended in RNase-free water and ligated sequentially to 5' (5'-TGGGAATTCCTCACTaaa) and 3' (5'-PuuuCTATCCATGGACTG-TidT-3') RNA/DNA adaptors (lower-case nucleotides, ribonucleotides; P, phosphate group; idT, inverted deoxy thymidine modification). Small RNAs were first ligated to the 5' adaptor using T4 RNA ligase (Amersham, Little Chalfont, UK) and the ligation product was ethanol precipitated, resolved on denaturing 15% polyacrylamide, gel eluted, ethanol precipitated, and ligated to the 3' adapter using T4 RNA ligase (Amersham). The final ligation product was ethanol precipitated, resolved on denaturing 15% polyacrylamide, gel eluted, ethanol precipitated, and used as a template in a reverse transcription reaction using an antisense primer (5'-CGAACATGTACAGTCCATGGATAG-3') and Superscript II RT (Invitrogen, Carlsbad, CA, USA). Resulting cDNAs were amplified by PCR using sense (5'-CAGCCATGGGAATTCCTCACTAAA-3') and antisense (5'-CGAACATGTACAGTCCATGGATAG-3') primers containing internal *EcoRI* and *NcoI* sites (underlined), respectively. Resulting PCR products were purified, digested with *EcoRI/NcoI*, and resolved on native 15% polyacrylamide. A gel slice containing DNAs of approximately 40–60 nt (based on oligonucleotide size standard) was excised and eluted in 0.3 M NaCl at 4°C for approximately 16 h. Eluted DNA was ethanol precipitated, resuspended in water, and ligated to *EcoRI/NcoI*-digested pGEM-T Easy vector (Promega, Madison, WI, USA). Following transformation, plasmid was purified from ampicillin-resistant colonies and sequenced.

RNA gel-blot analysis

Total RNA was resolved by electrophoresis on denaturing 15% polyacrylamide gel containing 7 M urea in TBE buffer (45 mM Tris-borate pH 8.0, 1.0 mM EDTA), electroblotted to Zeta-Probe membrane (Bio-Rad, Hercules, CA, USA) using a trans-blot transfer cell (Bio-Rad) for 1 h at 500 mA. Following transformation, the membrane was UV cross-linked and dried overnight. Radiolabelled probes were made by end-labelling of DNA oligonucleotides complementary to sRNA sequences with $\gamma^{32}\text{-ATP}$ using T4 polynucleotide kinase (NEB, Ipswich, MA, USA). Blots were prehybridized and hybridized using EZ-hybridization solution (Biological Industries, Beit-Haemek, Israel). Hybridization was performed at 40°C overnight. Blots were washed two or three times at 50°C with washing buffer ($2 \times \text{SSC}$, 0.2% SDS) and autoradiographed using a phosphorimager (Fuji). Densitometric analysis was carried out using the

IMAGEJ 1.33u program (National Institutes of Health) to assess the relative expression level of each sRNA in different samples.

Computational analyses

Small RNA sequences were subjected to BLASTN analyses against the *P. patens* EST database (<http://moss.nibb.ac.jp/blast/blast.html>); the miRNA Registry (<http://www.sanger.ac.uk/Software/Rfam>); the Arabidopsis Small RNA Project database (<http://asrp.cgrb.oregon-state.edu/db/search.html>); the EST and intergenic region of TAIR database (<http://www.arabidopsis.org/Blast>); and the NCBI nr database (<http://www.ncbi.nlm.nih.gov/BLAST>). Fold-back structures were predicted and their stability validated using the MFOLD program (<http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi>), default parameters (Zuker, 2003) and the RANDFOLD program, 999 randomizations and dinucleotide shuffling (Bonnet *et al.*, 2004). Target prediction was done by searching the *P. patens* EST database for sRNA complementary sequences allowing up to three mismatches, no gaps or deletions, using PATSCAN (Dsouza *et al.*, 1997).

Molecular cloning of PpSBP3

A cDNA library representing the entire life cycle of *P. patens* (H. Sommer, S. Höhmann and PH, Max Planck Institute for Plant Breeding Research, Cologne, Germany, unpublished data) was screened with a ^{32}P radioactively labelled probe according to standard protocols (Sambrook *et al.*, 1989). The probe was obtained after amplification from *P. patens* genomic DNA using a primer pair (MR07 5'-CAAATTGCCGACGTGAACCTGAGGACG-3' and MR09 5'-TGAGGAGCCCGACGAAGATTTG-3') designed according to a *P. patens* EST sequence containing an SBP-box. A full-length cDNA of *PpSBP3* resulting from this screen was subcloned, using the λNM1149 phage-specific primers MR14 5'-CCAGTCAACACTTACGCAAGAG-3' and MR15 5'-TCGCCTCCATCAACAACTTTC-3', into pCR2.1 Topo (Invitrogen), and subsequently sequenced.

Cleavage site mapping of PpSBP3

A modified procedure for RNA ligase-mediated rapid amplification of cDNA ends (5'-RACE) was followed with the GeneRacer Kit (Invitrogen) as described by Llave *et al.* (2002b). Total RNA was isolated from 7-day-old protonemata grown on a minimal medium with NH_4 tartrate. Poly(A)-mRNA was prepared using an Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA) and directly ligated (112 ng) to GeneRacer RNA Oligo adaptor without further modification. The GeneRacer Oligo dT-18 primer was used to prime cDNA synthesis with reverse transcriptase superscript II (Invitrogen). This cDNA was subjected to an amplification procedure with the GeneRacer 5' primer (5'-CGACTGGAGCAGGACTGA-3') and a 3' *PpSBP3*-specific primer (5'-GCAGATCGGTGAACCCGCGGTGCTCAC-3'). The conditions used for this amplification step were the same as those for gene-specific RACE recommended by the manufacturer. The 5'-RACE product was gel purified, cloned and sequenced.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Sequences of sRNAs cloned from *Physcomitrella patens*

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