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Research Article

Molecular Diversity of miR390-Guided Transacting siRNA Precursor Genes in Lower Land Plants: Experimental Approach and Bioinformatics Analysis

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Transacting siRNA loci (TAS3-like) of a particular plant species are usually represented by several gene families. PCR-based approach was used as a phylogenetic profiling tool to probe genomic DNA samples from representatives of evolutionary distant Bryophyta taxa, namely, class Bryopsida (subclasses Bryidae and Dicranidae) and class Sphagnopsida. We found relatives of all four *Physcomitrella patens* (subclass Funariidae) TAS3-like loci in subclasses Bryidae and Dicranidae. Only representatives of subclass Bryidae encoded TAS3-like genes belonging to *P. patens* TAS3a and TAS3d families. On the other hand, only the members of order Grimmiales (subclass Dicranidae) encoded gene relatives of *P. patens* TAS3c family. These data indicate that moss ta-siRNA families have been long conserved during land plant evolution. However, *P. patens* TAS3-like loci were detected neither in two *Sphagnum* species from the earliest diverged moss class Sphagnopsida, nor in the *Selaginella kraussiana* from the earliest extant tracheophyta lineage, Lycopodiopsida.

1. Introduction

Plants produce several distinct types of small RNAs that are predominantly 21 to 24 nucleotides in size. Small interfering RNAs (siRNAs) including heterochromatic siRNAs (hc-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), trans-acting siRNAs (ta-siRNAs), and secondary siRNAs are involved in transcriptional and posttranscriptional gene silencing [1–10]. Deep sequencing experiments have demonstrated that thousands of loci per genome generate these siRNAs, with 24 nt hc-siRNA loci being predominant [10, 11]. In contrast, microRNAs (miRNAs) are encoded by perhaps just several hundred loci, which are transcribed with RNA polymerase II (Pol II) to generate single-stranded precursors further processed into specific miRNAs. These precursors contain the mature miRNA sequence within an imperfect double-stranded RNA hairpin, which is processed in the nucleus by Dicer-like (DCL) protein DCL1 to give an imperfect RNA duplex with two-nucleotide 3′-overhangs on each strand. One strand derived from this duplex functions as a miRNA. In total, several dozens of plant miRNA families (hundreds of individual miRNA species) are currently identified and found to target mainly protein-coding mRNAs [2, 4, 9, 12, 13].

Endogenous ta-siRNAs arise from Pol II-transcribed genes and function in plants to guide cleavage of target mRNAs [16]. Biogenesis of ta-siRNAs requires RNA-dependent RNA polymerase 6 (RDR6), suppressor of gene silencing 3 (SGS3), and DCL4. Like other siRNAs and miRNAs, ta-siRNAs are methylated at 2′O of the 3′terminal nucleotide by HEN1, an RNA methyltransferase. The biogenesis pathway for ta-siRNAs is initiated by a site-specific cleavage of primary *TAS* transcripts by AGO proteins guided by a miRNA [7, 8, 17]. The processed primary transcript is then converted to a double-stranded (ds)RNA precursor by the activity of RDR6 in cooperation with SGS3. A DCL4 dicer activity then

catalyzes the precursor cleavage which occurs with 21-nt intervals starting from the end of precursor generated by miRNA-guided cleavage. Active ta-siRNAs, therefore, are accurately phased with respect to the site of miRNA-guided cleavage. The generated 21-nt ta-siRNAs further work as components of an RNA-induced silencing complex (RISC) to guide AGO-dependent cleavage of their target mRNAs. Recent studies have reported that some ta-siRNAs also work as cleavage guiders to cut ta-siRNA precursors transcribed from *TAS* genes, similarly to miRNA, and thus generate secondary ta-siRNAs with new specificity [17–20]. In *Arabidopsis thaliana*, eight ta-siRNA-generating loci have been identified that fall into four families (At*TAS1-4*). Most *TAS* gene transcripts have only a single miRNA target site (miR173 for *TAS1* and *TAS2* and miR828 for *TAS4*) positioned 5′ to the ta-siRNA-producing region, and the target site is cleaved by AGO1 slicer activity guided by the respective miRNA [21]. However, two miR390 binding sites (5′ and 3′ to the ta-siRNA encoding region) were shown to be necessary for the *TAS3* precursor RNA processing, that appeared to be dependent on specific interaction between AGO7 and miR390 [17–20]. In a current model, the biogenesis of At*TAS3*-derived ta-siRNAs is initiated by miR390-mediated cleavage of a capped and polyadenylated *TAS3* transcript at the 3′ binding site by the AGO7 slicer activity. The miR390-AGO7 complex interaction with a noncleavable 5′ binding site appears to be required for recruitment of SGS3 and RDR6 to produce dsRNA spanning the ta-siRNA precursor up to the 5′ binding site for miR390 (M. Pooggin, personal communication). Then DCL4, in cooperation with its binding partner DRB4, processes the resulting dsRNA into 21-nt duplexes starting from the miR390-cleaved terminus [17].

Although ta-siRNA genes (*TAS* genes) were first found in higher plants [3], their existence also in the moss *Physcomitrella patens* has been later reported [22, 23]. The *TAS3* family genes and miR390 have been identified in various plant species including eudicots, monocots, and gymnosperm, suggesting a conserved function of the encoded tasiARFs, which play an important role in auxin responses and plant development through posttranscriptional regulation of mRNAs coding for their target transcription factors, auxin response factor 3 (ARF3) and ARF4 [2, 8, 12, 24]. Interestingly, although all four identified *P. patens TAS3*-like loci code for tasiARF species homologous to those in flowering plants [22] that indicates their extreme evolutionary conservation, no sequenced *TAS3*-loci were reported in other lower land plants (namely, ferns, lycopods, and bryophytes). However, the data supporting ta-siRNAs formation were obtained for unicellular green algae *Chlamydomonas reinhardtii* [25]. Moreover, miR390 sequences were revealed in fern (*Ceratopteris thalictroides*) and moss *Polytrichum juniperinum* (class Polytrichopsida) that strongly suggests the presence of TAS3-like RNA precursors in these organisms [24].

Previously, we described the new method for identification of plant ta-siRNA precursors based on PCR with oligodeoxyribonucleotide primers mimicking miR390. The method was found to be efficient for dicotyledonous plants, cycads, conifers, and mosses [15]. In this study, PCR-based approach was used as a phylogenetic profiling tool to probe genomic DNA samples derived from *Selaginella kraussiana* (Lycopodiopsida) and 13 bryophyta species from classes Bryopsida (subclasses Bryidae and Dicranidae) and Sphagnopsida (*Sphagnum squarrosum* and *Sphagnum girgensohnii*). We failed to detect TAS3-like loci in *Selaginella kraussiana* and *Sphagnum sp.* Nevertheless, we found relatives of all four *P. patens* TAS3-like loci in Bryidae and Dicranidae species. These results indicate that at least some of ta-siRNA families have been long conserved during land plant evolution.

2. Materials and Methods

2.1. Plant Material. Moss specimens for 13 studied species listed in Table 1 and *Selaginella kraussiana* were taken from collections of the N. V. Cycin Main Botanic Garden of the Russian Academy of Sciences.

2.2. Analysis of Nucleic Acids. Total DNA was isolated from about 200 mg of plant material by Nucleospin Plant Kit (Macherey-Nagel) according to the protocol of the manufacturer. For amplification, the *Physcomitrella* TAS3-specific primers [15] were used: a forward primer Bryo TAS-P 5′-GRCGYTAYCCTCYTGAGCT-3′ and reverse primer Bryo TAS-M 5′-TAGCTCAGGAGTGATA(G/T)A(C/A)AA-3′. For PCR, 25–35 cycles were used for amplification with a melting temperature of 95°C, an annealing temperature of 58°C, and an extending temperature of 72°C, each for 30 seconds, followed by a final extension at 72°C for 3 min. PCR products were separated by electrophoresis of samples in 1.5% agarose gel and purified using the GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences). For cloning, the PCR-amplified DNA bands isolated from gel were ligated into pGEM-T (Promega). Cloned products were used as templates in sequencing reactions with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). DNA sequences were deposited at the NCBI data bank, and the accession numbers are shown in Table 1.

2.3. Computational Sequence Analysis. TAS3-like sequences identified in moss species were compared using multiple alignment tool at MAFFT (version 6). The MAFFT package is available at http://mafft.cbrc.jp/alignment/server/. Sequence similarities were additionally analysed by NCBI Blast at http://blast.ncbi.nlm.nih.gov/BlastAlign.cgi. The presence of open reading frames within sequenced amplification products was analysed at http://web.expasy.org/translate/.

3. Results and Discussion

Previously, four ta-siRNA loci targeted by miR390 were found in moss *Physcomitrella patens* and referred to as PpTAS3a-d. All four loci contain 5′ and 3′ miR390-target sites, and ta-siRNAs derived from these moss loci regulate, similarly to those encoded by the angiosperm TAS3 loci, ARF genes and, additionally, target mRNA of AP2 transcription factors [22]. To apply the approach based on PCR amplification [15] to TAS3-like loci of lower land plants, we designed degenerated primers BryoTAS-P and Bryo TAS-M

Table 1: Bryophyta species studied and data on their sequenced TAS3-like loci.

Class/subclass	Order	Species	Clone	Length, bp	Accession number	*P. patens* family
Bryopsida/Dicranidae	Grimmiales	*Coscinodon humilis*	20-Chu	234	HQ709423	TAS3c
		C. humilis	16-Chu	228	HQ709422	- - -"- - -
		Schistidium elegantulum	24-Sce	229	HQ709424	- - -"- - -
		Grimmia cribrosa	43-Gcr	228	HQ709421	- - -"- - -
	Dicranales	*Fissidens taxifolius*	17-Fit	192	HQ709425	TAS3b
Bryopsida/Bryidae	Orthotrichales	*Orthotrichum pumilum*	5-Opu	199	HQ709419	- - -"- - -
		O. pumilum	24-Opu	246	HQ709420	TAS3a/d
	Hypnales	*Brachythecium latifolium*	47-Br	199	FJ804748	TAS3b
		B. latifolium	50-Br	255	FJ804747	TAS3a/d
		B. albicans	37-Bal	199	HQ709414	TAS3b
		Homalothecium philippeanum	7-Hp	199	HQ709415	- - -"- - -
		Oxyrrhynchium hians	25-Oxy	199	HQ709418	- - -"- - -
		Pylaisia polyantha	10-Pyp	199	HQ709416	- - -"- - -
		P. polyantha	11-Pyp	253	HQ709417	TAS3a/d
	Hookeriales	*Hookeria lucens*	49-H	190	FJ804749	TAS3b
Sphagnopsida	Sphagnales	*Sphagnum squarrosum*	no	—	—	—
		S. girgensohnii	no	—	—	—

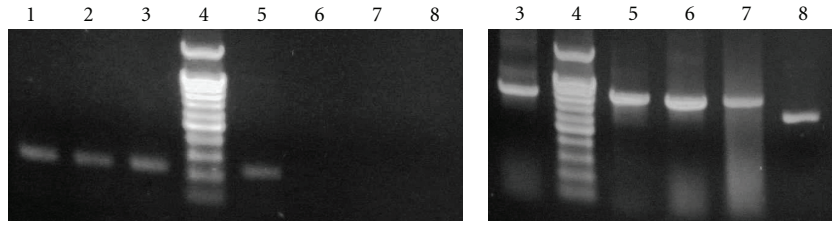

Figure 1: Analysis of PCR products in 1.5% agarose gel. Amplification of genomic DNA sequences flanked by miR390 sites. (a) PCR products were obtained on genomic DNAs with moss-specific primers. *Orthotrichum pumilum* (1), *Coscinodon humilis* (2), *Pylaisia polyantha* (3), *Homalothecium philippeanum* (5), *Sphagnum girgensohnii* (6), *Sphagnum squarrosum* (7), and *Selaginella kraussiana* (8). (4), DNA size markers including bands ranging from 100 bp to 1000 bp with 100 bp step and 1500 bp band (Sibenzyme). (b) PCR products were obtained on genomic DNAs with the internal transcribed spacer-specific (ITS2-specific) primers [14] as a control of genomic DNA preparation quality. Lane numbering corresponds to those in (a).

complementary to miR390-target sites of PpTAS3a-d [22]. These primers were used for PCR on total DNA isolated from mosses belonging to classes Bryopsida and Sphagnopsida as well as from the lycopod *Selaginella kraussiana*.

PCR amplification of chromosomal DNA from representatives of subclasses Bryidae and Dicranidae resulted in synthesis of major bands of 200–250 bp (Figure 1 and Table 1). Cloning and sequencing of the obtained DNA bands revealed that the amplified sequences similarly to *P. patens* contained well-conserved TAS3 region composed of AP2-specific ta-siRNA sequence followed by ARF-specific sequence and located between miR390 target sites corresponding to PCR primers [22]. Moreover, amplified sequences showed obvious similarity to TAS3-like genes from *P. patens* plants (Figures 2 and 3) as it was revealed by bioinformatic analysis of the putative TAS3-like sequences using multiple sequence alignment tool MAFFT. NCBI Blast revealed that *Orthotrichum pumilum* clone 24-Opu TAS3-like sequence showed 71% identity to the *P. patens* locus TAS3a genomic sequence, *Brachythecium latifolium* clone 50-Br sequence showed 72% identity to the *P. patens* locus TAS3d genomic sequence, *Fissidens taxifolius* clone 17-Fit sequence showed 76% identity to the *P. patens* locus TAS3b genomic sequence, and *Coscinodon humilis* clone 20-Chu showed almost 70% identity to the *P. patens* locus TAS3c genomic sequence.

As a rule ta-siRNA loci of a particular plant species are represented by several gene families. For example, four ta-siRNA loci targeted by miR390 were found in *P. patens* (see above), and three miR390-dependent TAS3 loci were identified in *A. thaliana* [12, 17]. It was reasoned that multiple TAS3-like loci might be paralogs derived by duplication of an ancestral locus and subsequent divergence of its copies, and perhaps the areas of TAS3-like loci producing biologically relevant ta-siRNAs may have been preferentially conserved

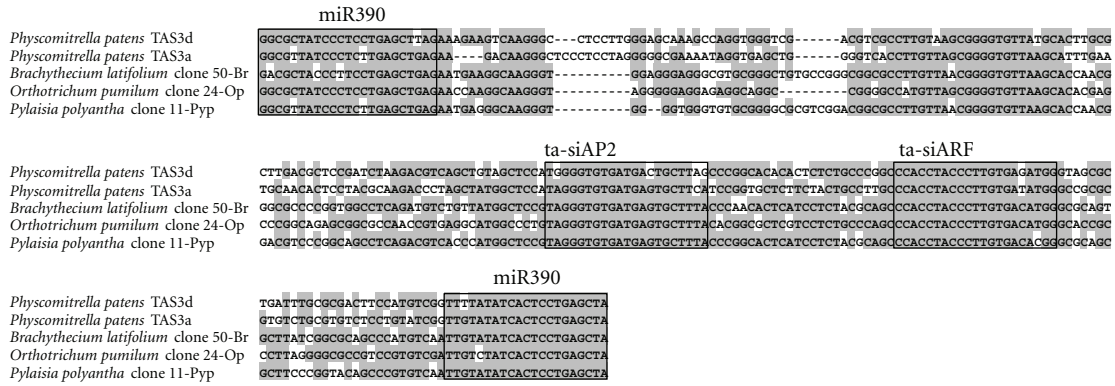

Figure 2: Nucleotide sequence alignment of ta-siARF loci of *P. patens* TAS3a and d and moss TAS3-like sequences determined in this and previous [15] papers. Shading shows residues identical for the given position in most sequences.

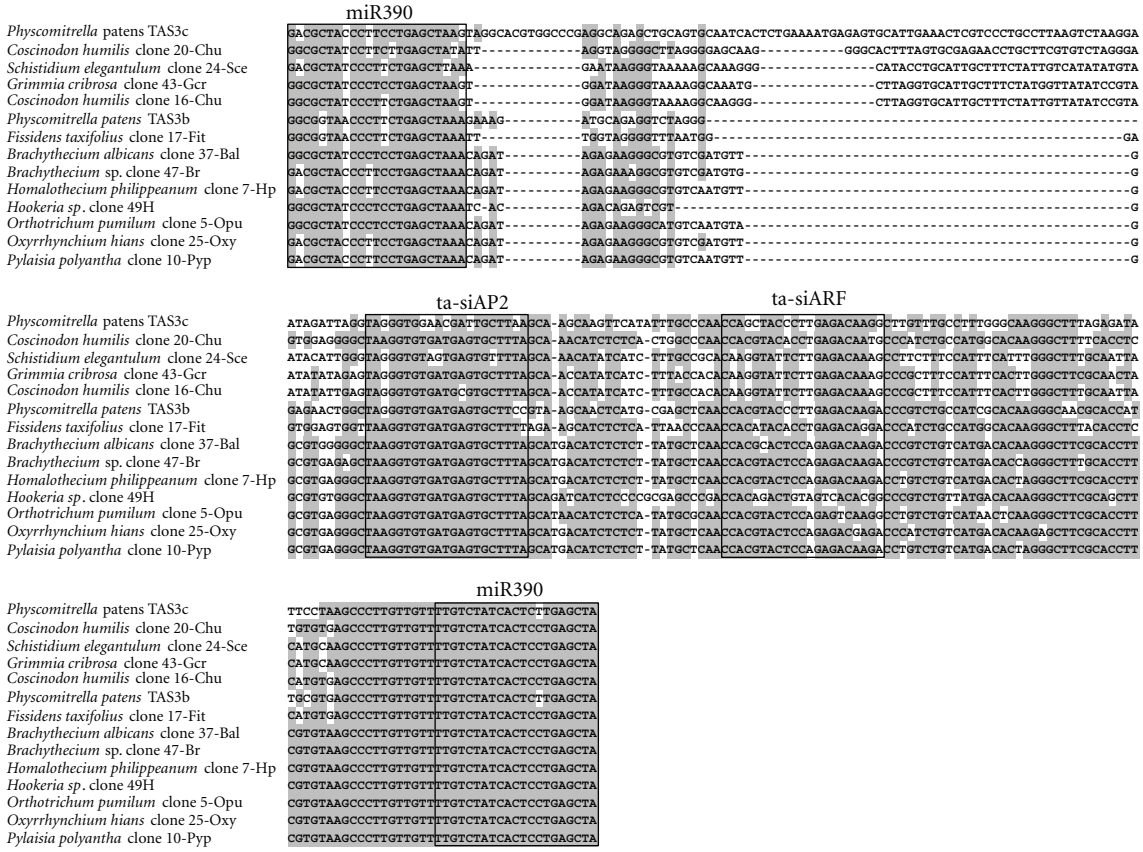

Figure 3: Nucleotide sequence alignment of ta-siARF loci of *P. patens* TAS3b and c and moss TAS3-like sequences determined in this paper and previous [15] papers. Shading shows residues identical for the given position in most sequences.

[20, 22]. However, despite obvious progress in phylogenetic studies of miRNA precursor evolution in plants [2, 4, 9, 12, 27], little is known about TAS gene molecular evolution. In this paper, we attempted to estimate the diversity of TAS3-like genes in moss species from class Bryopsida (subclasses Bryidae and Dicranidae) and subdivide them according to gene families known for *P. patens*—a representative of Funariidae, another subclass of Bryopsida (Figure 4). Sequencing of clones from major amplified DNA bands showed that *Brachythecium latifolium*, *Coscinodon humilis*, *Orthotrichum pumilum,* and *Pylaisia polyantha* encode at least two types of miR390-dependent AP2/ARF-targeting TAS loci demonstrating sequence similarity to different families of TAS loci characterized for *Physcomitrella patens* (Figures 2 and 3). Totally, we investigated 11 *Bryopsida* moss species (Table 1). Six of them belong to orders *Hookeriales* and *Hypnales*, which together comprise more than 25% of all known moss species [14]. Subclass *Dicranidae* (totally 4 species studied) was mainly represented by plants from order *Grimmiales* (Figure 4 and Table 1).

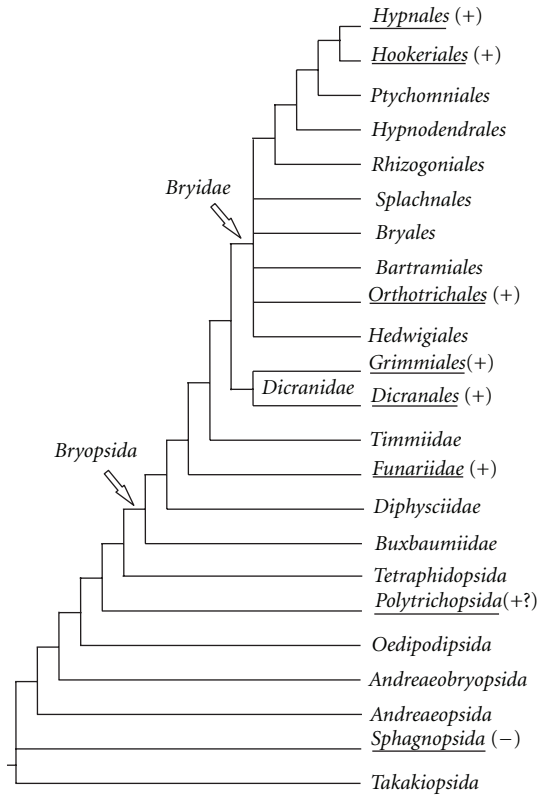

Figure 4: Synoptic consensus view on phylogeny of major Bryophyta taxa based on recent molecular analyses, adopted from Figure 3 of Shaw et al. [26] with modifications. Taxa discussed in the current study are underlined. Taxa from Hypnales (upper) to Dicranales (lower) represent orders; taxa from *Timmiidae* (upper) to *Buxbaumiidae* (lower) represent subclasses; taxa from Tetraphidopsida (upper) to Takakiopsida (lower) represent classes. (+) indicates the encoding of miR390-guided TAS3-like genes, and (−) indicates the possible absence of miR390-guided TAS3-like genes. Arrows indicate branching points showing monophyletic origin of class Bryopsida and subclass Bryidae.

P. patens loci TAS3a and TAS3d encode rather closely related TAS genes (Figure 1) [15]. However, we found that TAS3d family is unique among TAS genes because it overlaps a protein-coding gene ("GeneID: 5915198"). TAS3d precursor RNA is complementary to the 5′-terminal region of the mRNA (NCBI accession number XM_001751798) and one intron. The encoded protein of unknown function (NCBI accession number XP_001751850) is 292 amino acids in length and has no closely related sequences in other plants. Inspection of nucleotide sequences of *P. patens* TAS3a and related loci described in this paper (*Brachythecium latifolium* clone 50-Br, *Orthotrichum pumilum* clone 24-Opu, and *Pylaisia polyantha* clone 11-Pyp) (Table 1) at Expasy web service (see Section 2) showed that complementary sequences of these four loci cannot encode protein because of the lack of initiation AUG codons or the presence of multiple stop codons although all of them include sequences corresponding to the intron of *P. patens* TAS3d-overlapping protein-coding gene 5915198 (data not shown). Despite this difference, sequence alignment shows obvious similarity between all five TAS precursor genes shown in Figure 2. Most conserved area represents the region including AP2-specific and ARF-specific ta-siRNA sequence blocks and the spacer region between them (Figure 2). It was experimentally shown that this spacer also codes for ta-siRNA in *P. patens* [23]. Our BLAST analysis at NCBI showed that both complementary sequences of the AP2-ARF spacer can target *P. patens* mRNAs coding for uncharacterized proteins XP_001780327 (186 amino acids in length) and XP_001780322 (382 amino acids in length). There are 3.5 mismatches (counting G:U wobbles as 0.5 mismatches) between these ta-siRNAs from TAS3d locus and each of the targeted mRNAs, which is just at the cutoff for confident computational target prediction [28]. The sequences from *Brachythecium latifolium* clone 50-Br, *Orthotrichum pumilum* clone 24-Opu, and *Pylaisia polyantha* clone 11-Pyp show 6–8 mismatches that can reflect significant evolutionary distance (Figure 4) between these moss species and *P. patens*.

Analysis of the sequence alignments shown in Figures 1 and 2 suggests that only representatives of subclass Bryidae encode TAS3-like genes belonging to *P. patens* TAS3a and TAS3d families (Table 1). On the other hand, only the Dicranidae species code for the genes closely related to *P. patens* TAS3c family (Figure 2 and Table 1). To support this observation as a conclusive general view on peculiarities of TAS loci evolution in mosses, more detailed analysis of TAS gene diversity is required. Importantly, the activity in this direction can be inferred from the previously published data which strongly suggested the presence of miR390 species and, respectively, TAS3-like RNA precursors in the earlier diverged moss class Polytrichopsida (Figure 4) [24].

Unlike mosses of class Bryopsida showing at least one visible DNA band after PCR amplification, genomic DNAs of the Sphagnopsida mosses from the earliest diverged (except *Takakia*) Bryophyta lineage (Figure 4), namely, *Sphagnum squarrosum* and *Sphagnum girgensohnii,* showed no PCR products with *P. patens*-specific (Figure 1) and dicot-specific miR390-mimicking primers [15] (data not shown). The same negative result was obtained for a lycopod *Selaginella kraussiana* (Figure 1) that is in accordance with the data reported previously for *Selaginella uncinata* [24]. This finding was intriguing due to the fact that at least some miRNA families including miR390 have remained essentially unchanged since the last common ancestor of mosses (*P. patens*) and flowering plants [8, 17, 24]. However, during the preparation of the paper, we learned that the completely sequenced genome of *Selaginella moellendorffii* belonging to the earliest extant tracheophyta lineage having common ancestors with mosses contains no DCL4, RDR6, and MIR390 loci required for the biogenesis of ta-siRNAs [29]. Their absence suggests that ta-siRNA-dependent processes in plants are regulated differently in distinct plant lineages and possibly reflects the independent evolutionary history (retention or loss) of the TAS genes in separate taxons. Moreover, our results support that purifying selection is one of the main evolutionary forces acting on TAS3-like genes, maintaining lower levels of sequence divergence in mature ta-siRNAs (AP2-specific and ARF-specific sequences of precursors) than in their flanking regions (Figures 2 and 3). Although we

identified several mutations in the functional regions of the candidate ta-siRNA genes in mosses, such as in ta-siARF, ta-siAP2, and the miR390 binding sites (Figures 2 and 3), it does not necessarily indicate a direct consequence of the positive selection events. Further experiments involving identification of resulting phenotypic changes are needed to reveal and prove the events of positive selection.

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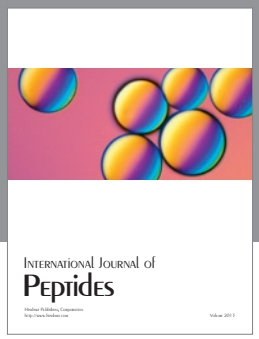

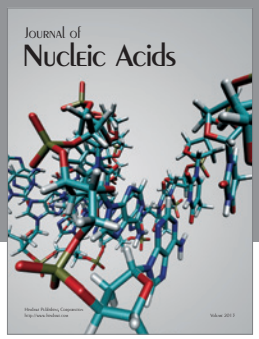

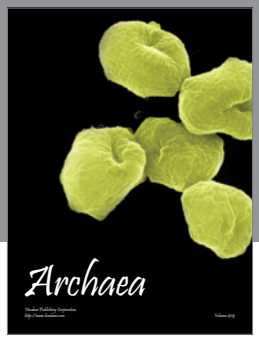

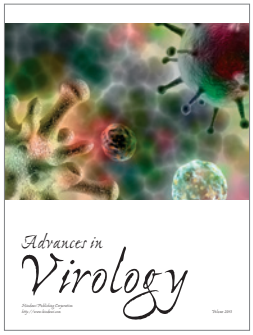

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