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Short communication

3′ RNA ligase mediated rapid amplification of cDNA ends for validating viroid induced cleavage at the 3′ extremity of the host mRNA

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ABSTRACT

5′ RNA ligase-mediated rapid amplification of cDNA ends (5′ RLM-RACE) is a widely-accepted method for the validation of direct cleavage of a target gene by a microRNA (miRNA) and viroid-derived small RNA (vd-sRNA). However, thi\s method cannot be used if cleavage takes place in the 3′ extremity of the target RNA, as this gives insufficient sequence length to design nested PCR primers for 5′ RLM RACE. To overcome this hurdle, we have developed 3′ RNA ligase-mediated rapid amplification of cDNA ends (3′ RLM RACE). In this method, an oligonucleotide adapter having 5′ adenylated and 3′ blocked is ligated to the 3′ end of the cleaved RNA followed by PCR amplification using gene specific primers. In other words, in 3′ RLM RACE, 3′ end is mapped using 5′ fragment instead of small 3′ fragment. The method developed here was verified by examining the bioinformatics predicted and parallel analysis of RNA ends (PARE) proved cleavage sites of *chloride channel protein CLC-b-like* mRNA in *Potato spindle tuber viroid* infected tomato plants. The 3′ RLM RACE developed in this study has the potential to validate the miRNA and vd-sRNA mediated cleavage of mRNAs at its 3′ untranslated region (3′ UTR).

RNA interference (RNAi, RNA silencing) regulates gene expression both transcriptionally and post-transcriptionally (Baulcombe, 2004). Either the double-stranded RNA (dsRNA) or the self-complementary RNA triggers RNA silencing through the activity of the DICER-*like* (DCL) *RNase* III-type ribonucleases, and result in the production of small interfering RNAs (siRNA). These small RNAs (sRNA) comprise the sequence-specific effectors that regulate gene expression (Gregory et al., 2008). In plants and animals, microRNAs (miRNA) regulate gene expression by RNAi. This regulation is triggered by the binding of miRNA to its target RNA, resulting in either translational repression or endonucleolytic cleavage (Brodersen et al., 2008; Llave, 2002).

Viroids are plant pathogenic single-stranded RNA (ssRNA) molecules of 246–401 nucleotides (nts) in length. To date, viroids are not known to code for any protein; hence, they must completely rely on host factors for their replication (Ding, 2009). Due to their high internal base pairing and RNA–RNA mode of replication, viroids are the elicitors of the host defense system via RNA silencing. As a result, viroid derived sRNA (vd-sRNA), which are 21–24 nts in size, are often recovered from viroid infected host plants (Adkar-Purushothama et al., 2015b; Diermann et al., 2010; St-Pierre et al., 2009; Tsushima et al., 2015). Previously, it has been shown that viroid derived small RNAs are able to induce the cleavage of endogenous mRNAs through RNAi (Adkar-Purushothama et al., 2017,2015a; Avina-Padilla et al., 2015; Eamens et al., 2014; Navarro et al., 2012).

Several computer-based prediction programs scan plant genomic or cDNA sequences against either the miRNA or vd-sRNA for the complementary sequence to a potential target, using predefined rules (Rhoades et al., 2002). However, these approaches fail to distinguish between false and true targets. Hence, biological validation of algorithm predicted target sites are very important. In this scenario, high-throughput 5′ RLM RACE techniques, such as the parallel analysis of RNA ends (PARE), the degradome approach and the genome-wide mapping of uncapped and cleaved transcripts (GMUCT), have gained importance as these methods use global sampling (Addo-Quaye et al., 2008; German et al., 2008; Gregory et al., 2008). Due to its high cost, it is not convenient to many laboratories that are searching for single targets. Conventionally, 5′ RNA ligase mediated rapid amplification of cDNA ends (5′ RLM RACE) is the widely accepted technique to confirm the direct cleavage of a target mRNA by a miRNA (Thomson et al., 2011). The same technique has been applied to validate the cleavage of predicted target mRNAs in viroid infected plants (Adkar-Purushothama et al., 2015a; Eamens et al., 2014; Navarro et al., 2012). As 5′ RLM RACE involves adding RNA adapter to the 5′ end followed by nested PCR using gene specific primers from the 3′ end of the cleavage site, this technique is not of much use if the predicted cleavage is in 3′ end of the gene sequence. This is due to the fact that cleavage at 3′ extremity of

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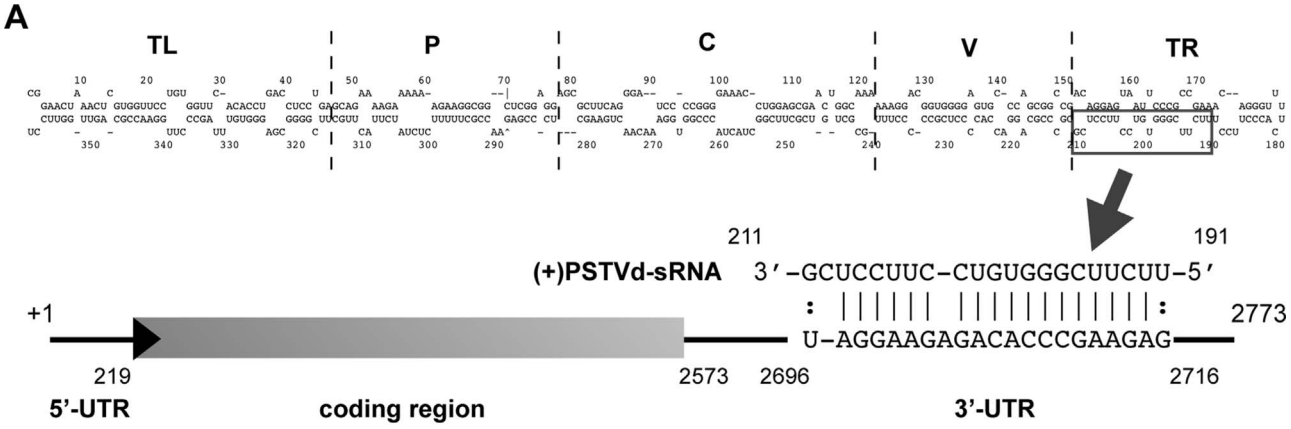
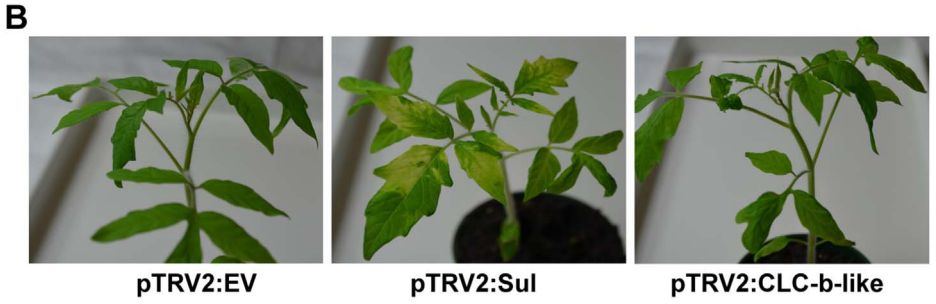
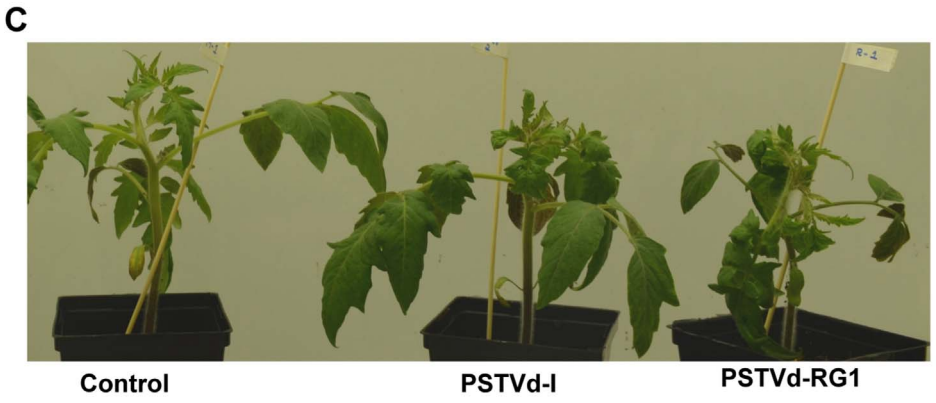
the target RNA gives insufficient sequence length to design nested PCR primers for 5′ RLM RACE. Hence, in the present study we have developed 3′ RLM RACE to enable validation that the target mRNA cleavage occurs at the 3′ end, e.g. 3′ UTR. In this method, an RNA adapter is ligated to the 3′ end of the cleaved RNA. This 3′ adapter is used to map the 3′ end of the 5′ fragment instead of mapping small 3′ fragment.

In previous investigation for possible vd-sRNA binding 3′UTR of targets in tomato transcriptome datasets using the psRNATarget web-based tool (Adkar-Purushothama et al., 2017), we detected possible interaction of sRNA derived from the lower terminal right (TR) domain







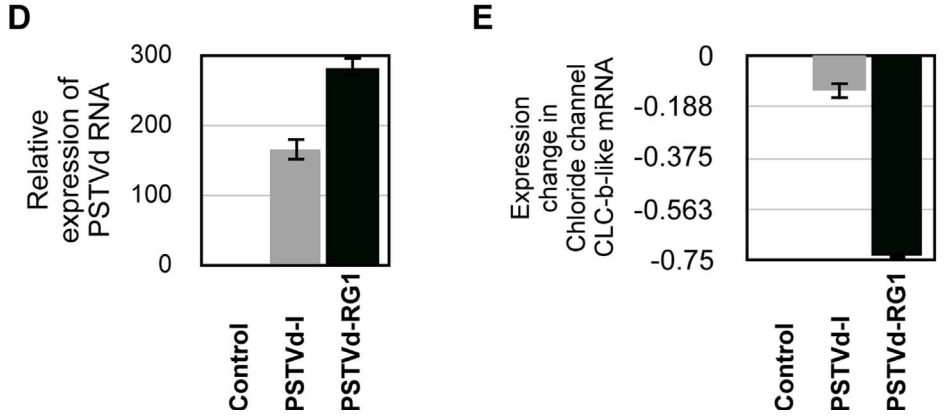


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Fig. 1. Schematic representation of predicted vd-sRNA/target duplexes and the effect of the PSTVd variants on the *chloride channel protein CLC-b-like* mRNAs.
(A) Upper panel shows the secondary structure of PSTVd showing the five functional/structural domains, i.e., Terminal left (TL), Pathogenecity (P), Central (C), Variable (V) and Terminal right (TR) domain. The box on the lower TR domain indicates PSTVd-sRNA which was predicted to target *chloride channel protein CLC-b-like* mRNAs. Lower panel shows the predicted interactions between the PSTVd-sRNAs with the 3′UTR of the *chloride channel protein CLC-b-like* mRNA. The sequences are shown in the complementary polarity. (B) Genes in the tomato plants knock-down by a VIGS using the pTRV1 vector in combination with either the pTRV2 empty vector, or with its derivatives. After 3-weeks post infiltration plants exhibited phenotypic alterations. pTRV:EV, pTRV empty vector inoculated plants (control); pTRV2:Sul, plants inoculated with pTRV2:Sul; and, pTRV-CLC-b-like, the plant infiltrated with pTRV2:CLC-b-like. Both the PSTVd-I and PSTVd-RG1 variants were inoculated into tomato plants. (C) At 21-dpi, PSTVd variants inoculated plants showed disease symptoms when compared to mock inoculated plants. Total RNA extracted from tomato plants at 21-dpi were used to monitor the (D) PSTVd titer and (E) the effects of the PSTVd variants on the levels of the *chloride channel protein CLC-b-like* mRNAs. The expression change is presented on a $\log_2$ scale. Each experiment was performed at least three times with true biological replicates. The error bars indicate SD.

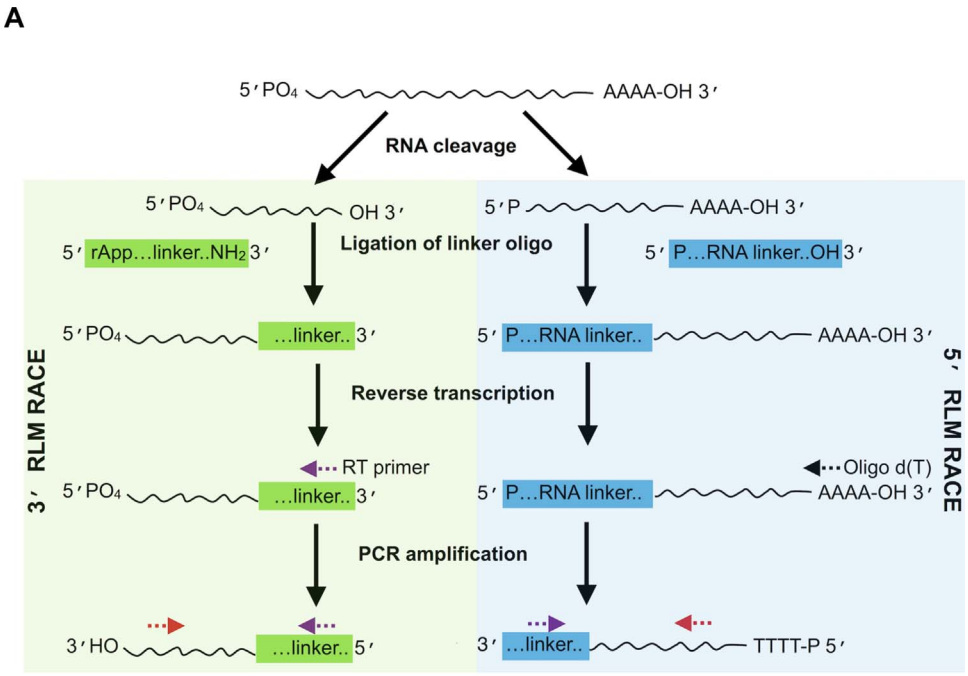
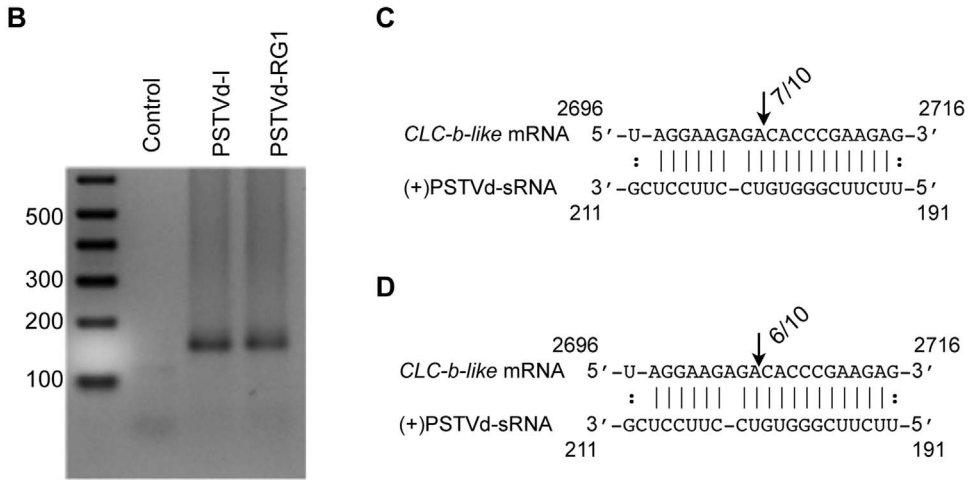


Fig. 2. 3′ RLM RACE and the analysis of RISC-Mediated Cleavage of the *chloride channel protein CLC-b-like* mRNA by 3′ RLM RACE.
(A) Flow chart illustrating the details of both the 3′ and 5′ RLM RACE experiments. In case of 3′ RLM RACE, a 3′ blocked adapter is added to the 3′ end of the cleavage RNA while an RNA adapter is added to the 5′ end of the cleaved RNA in 5′ RLM RACE. Adapter linked RNAs were subjected to RT followed by nested PCR using specific primers. (B) Total RNA was purified from mock-infected tomato plants, or plants infected with PSTVd-I or PSTVd-RG1, as indicated. Nested PCR products obtained from 3′ RLM RACE using primers targeting the *chloride channel protein CLC-b-like* mRNA were separated by 2.0% agarose gel electrophoresis. The *chloride channel protein CLC-b-like* mRNA/vd-sRNA duplexes predicted to be formed by the sRNA derived from the (C) PSTVd-I, and (D) PSTVd-RG1 variants. The arrows indicate the 3′ termini of *chloride channel protein CLC-b-like* mRNA fragments isolated from the PSTVd-infected plants, as identified by 3′ RLM-RACE products, with the frequency of clones shown (e.g. 7/10, indicates that 7 cleavage products were found out of 10 analyzed clones). Sequences are shown in the complementary polarity.



of the genomic strand of *Potato spindle tuber viroid* i.e. (+) PSTVd) with the 3′UTR of the *chloride channel protein CLC-b-like* mRNA (GenBank Acc. No. 115 XM_004233252; Fig. 1A, [Dai and Zhao, 2011]). Details of target prediction is described elsewhere (Adkar-Purushothama et al., 2017). The *chloride channel protein CLC-b-like* mRNA encodes for a chloride channel, which is an anion transporter (von der Fecht-Bartenbach et al., 2010). Chloride channels are involved in physiological functions such as cell volume regulation, stabilization of the membrane potential, control of electrical excitability, signal transduction, *trans-epithelial* transport and the acidification of intracellular organelles. These diverse functions require the presence of many distinct chloride channels that are differentially expressed and regulated by various stimuli, including intracellular messengers (calcium, cyclic AMP), *trans-membrane* voltage, extracellular ligands, and pH (Jentsch and Günther, 1997). The knock-down assay of *chloride channel protein CLC-b-like* mRNA in tomato plants using virus induces gene silencing (VIGS) technique was performed as described by Adkar-Purushothama et al. (2017) in order to verify the role of target gene on host's phenotype. pTRV2:Sul, which is capable of targeting suphur gene (encoding *magnesium-chelatase*) mRNA (*Sul*), was used as a positive

control, while a negative control was maintained by agro-infiltrating the plants with empty pTRV2 vector (pTRV2:EV). Approximately at 3 weeks post infiltration, pTRV2:CLC-b-like (targeting *chloride channel protein CLC-b-like* mRNA) agroinfiltrated plants exhibited mild stunting and leaf curling while positive control plants showed yellowish leaves compared to the control plants (Fig. 1B). In order to verify the effect of PSTVd infection on the *chloride channel protein CLC-b-like*, bioassays were performed in tomato plants using PSTVd-intermediate (PSTVd-I) and PSTVd-RG1 variants. Upon infection, the tomato plants inoculated with PSTVd variants exhibited disease symptoms such leaf curling and stunted growth (Fig. 1C). In order to monitor the accumulation of viroids and to evaluate the effect of viroid infection on *chloride channel protein CLC-b-like* mRNA, leaf samples were collected at 21-dpi and used to extract total RNA. The levels of viroids and *chloride channel protein CLC-b-like* mRNAs were then evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) on this total RNA as described previously (Adkar-Purushothama et al., 2017). As presented in Fig. 1D, at 21-dpi, PSTVd-RG1 accumulated much higher than that of the PSTVd-I. Similar analysis on *chloride channel protein CLC-b-like* mRNA revealed that PSTVd-RG1 inoculated plants exhibited higher level of down-regulation than that of PSTVd-I inoculated plants (Fig. 1E). Taken together, these data suggest that PSTVd infection has the direct relation with down-regulation of predicted target mRNA.

Though 5′ RLM RACE is the widely-accepted technique to confirm the direct cleavage of a target mRNA by a miRNA, it was not the best choice to validate the cleavage of *chloride channel protein CLC-b-like* mRNA in viroid infected plants due to the fact that predicted cleavage site is located close to the end of the mRNA sequence. As the terminus is located just 60 nts away from the predicted cleavage site, it is challenging to design nested PCR primers required for the amplification of cDNAs in 5′ RLM RACE. Hence, we developed a 3′ RLM RACE. In this strategy, an oligonucleotide adapter having 5′ adenylated, 3′ blocked oligonucleotide was ligated to the free 3′- hydroxyl end (3′-OH) of a cleaved RNA. The products were used for cDNA synthesis with oligonucleotide adapter specific primer followed by nested PCR amplification. Fig. 2A presents a schematic overview of 3′ RLM RACE and its comparison with 5′ RLM RACE.

In order to verify the RISC mediated cleavage of *chloride channel protein CLC-b-like* mRNA by 3′ RLM RACE, a 5′ adenylated, 3′ amine containing oligodeoxynucleotide universal miRNA cloning linker (New England Biolabs, Inc, MA, USA) was ligated to the free 3′-hydroxyl end of a cleaved RNA. Specifically, 10 μg of total RNA was mixed with a universal miRNA cloning linker in the absence of ATP and were incubated for 2 h at 37 °C in the presence of T4 RNA *ligase* I. The ligation product was reverse transcribed using a linker specific reverse primer. The cDNA products were subsequently amplified with nested PCR using *chloride channel protein CLC-b-like* mRNA primers (Table 1). The nested PCR products were separated by 2.0% agarose gel electrophoresis (Fig. 2B), and the band corresponding to the expected amplicon was eluted using the spinX column according to the manufacturer's instruction (E & K Scientific Products, Inc). The purified products were cloned into the pGEM-T easy vector (Promega) and sequenced commercially (http://www.sequences.crchul.ulaval.ca). The sequences were analyzed with CLC Free Workbench version 4.6 software (http://www.clcbio.com/index.php?id=28). The cDNA clones from the *chloride channel protein CLC-b-like* transcripts were aligned to detect the 3′ termini. Eight out of ten analyzed *chloride channel protein CLC-b-like* transcripts obtained for PSTVd-I inoculated plants had 3′ termini identical to the predicted cleavage site while seven out of ten clones had 3′ termini identical to the predicted cleavage site from the plants inoculated with PSTVd-RG1 (Fig. 2C,D). No PCR amplification was obtained when similar experiments were performed with RNA preparations obtained from mock-inoculated plants, indicating specific cleavage of the *chloride channel protein CLC-b-like*. Analysis of parallel amplification of RNA ends (PARE) library obtained for control (GEO Acc. No. GSE88707), PSTVd-I (GEO Acc. No. GSE70037), and PSTVd-RG1 (GEO Acc. No. GSE70062), inoculated tomato plants were in agreement with here obtained 3′ RLM RACE results, further validating the efficacy of the developed method (Adkar-Purushothama et al., 2017).

Both in plants and animals, the efficient RISC-mediated cleavage of a target by miRNA requires perfect or near perfect matches in the 5′ seed region as well as in the cleavage site (Fig. 3). Nonetheless, few mismatches are allowed in 3′ compensatory end of the miRNA:target duplex (Schwab et al., 2005). The predicted vd-sRNA:*chloride channel protein CLC-b-like* mRNA duplex had two mismatches, i.e. in the first and last nucleotide of the RNA/RNA duplex (the wobble base pairing located at position 1 of vd-sRNA, starting from the 5′ end and in the 3′ compensatory region [Fig. 1]). Although the RT-qPCR assays on total RNA extracted from PSTVd variants infected plants revealed the suppression in the expression of *chloride channel protein CLC-b-like* compared to that of control plants, it will not validate the cleavage of predicted target site by direct interaction of vd-sRNA. Hence, 5′ RLM RACE is an obvious choice given the credibility that it has been widely accepted for proving the RISC mediated cleavage of target miRNAs (Adkar-Purushothama et al., 2015a; Eamens et al., 2014; Navarro et al., 2012; Thomson et al., 2011). However, this was not able to prove that the vd-sRNA induced cleavage of *chloride channel protein CLC-b-like* mRNA, as the cleavage was predicted to take place in the 3′ extremity of the gene. This allows no, or only little space, to design primers for amplification of 5′ RNA ligated products by nested PCR. Hence, we developed a method in which a linker was added to the 3′ end of the cleaved RNA to enable use of the 5′ region of gene to design primers for nested PCR amplification (Fig. 2A).

In 3′ RLM RACE, an oligonucleotide adapter having 5′ adenylated and 3′ amine group was used. The presence of 3′-NH_2 blocks the self-ligation of the oligonucleotide, circularization as well as ligation to RNA at the 5′ end (Lau et al., 2001). Addition of ligase enzyme to the reaction mixture covalently ligates pre-activated (5′ adenylated) oligonucleotide adapter to the free 3′-hydroxyl group (OH) end of another single stranded RNA in the absence of ATP. Since RISC mediated cleavage results in the formation of free 3′-OH RNA molecules that allows the 5′ adenylated adapter to form covalent bond in the presence of ligase enzyme. Conventionally in order to determine 3′ sequence of

Table 1
Details of the primers used for the 3′ RLM-RACE experiment used to prove RISC-mediated cleavage site of the *chloride channel protein CLC-b-like* mRNA.

Primer name	Target gene	Primer sequence (5′-3′)	Primer binding site	Expected amplicon size (bp)
Adapter[a]		rAppCTGTAGGCACCATCAAT–NH_2	Adapter	
3′ RACE R[b]		AATGATACGGCGACCACCGACAGATTGATGGTGCCTAC		
3′ RACE nR		ACCGACAGATTGATGGTGCC		
CLC-b-RACE F	*CLC-b-like*	GCCACATGCTCATAGTACCG	2431–2450[c]	297
CLC-b-RACE nF		GCACTGAATCTCTCCGGAG	2567–2585[c]	144

Underlined nucleotide represents overlapping sequence which was used to design outer and inner primer for nested PCR.
[a] Universal miRNA Cloning Linker (New England Biolabs).
[b] RT and reverse primer for first round of PCR.
[c] GenBank Acc. No. XM_004233252.

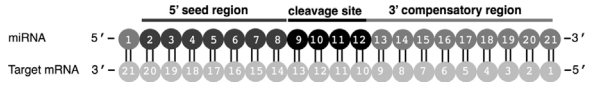


Fig. 3. Schematic diagram showing the miRNA:target mRNA complex.

the given mRNA, a known sequence adapter is added to the poly(A) tail by RT followed by PCR amplification using gene specific 5′ forward primer and adapter specific 3′ reverse primers. Since, cleavage of mRNA at 3′UTR not only removes poly(A) region, it gives insufficient sequence length to design primers for 5′ RLM RACE. In this scenario, the 3′ RLM RACE designed for the analysis of the cleavage of the 3′UTR of *chloride channel protein CLC-b-like* mRNA is a very interesting protocol for the scientific community which is looking for sequence of the 3′ end of the RNA that lacks poly(A) tail or is predicted to cleave near the end of 3′ termini giving less room for performing 5′ RLM RACE.

Acknowledgments

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