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CHLOROPLAST MRNAS ARE 3′ POLYURIDYLYLATED IN THE GREEN ALGA *PITHOPHORA ROETTLERI* (CLADOPHORALES)[1]

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Species within the green algal order Cladophorales have an unconventional plastome structure where individual coding regions or small numbers of genes occur as linear single-stranded DNAs folded into hairpin structures. Another group of photosynthetic organisms with an equivalently reduced chloroplast genome are the peridinin dinoflagellates of the Alveolata eukaryotic lineage whose plastomes are mini-circles carrying one or a few genes required for photosynthesis. One unusual aspect of the Alveolata is the polyuridylylation of mRNA 3′ ends among peridinin dinoflagellates and the chromerid algae. This study was conducted to understand if an unconventional highly reduced plastome structure co-occurs with unconventional RNA processing. To address this, the 5′ and 3′ mRNA termini of the known chloroplast genes of *Pithophora roettleri* (order Cladophorales) were analyzed for evidence of post-transcriptional processing. Circular Reverse Transcriptase PCR (cRT-PCR) followed by deep sequencing of the amplicons was used to analyze 5′ and 3′ mRNA termini. Evidence of several processing events were collected, most notably the 3′ termini of six of the eight genes were polyuridylylated, which has not been reported for any lineage outside of the Alveolata. Other processing events include poly(A) and heteropolymeric 3′ additions, 5′ primary transcript start sites, as well as the presence of circularized RNAs. Five other species representing other green algal lineages were also tested and poly(U) additions appear to be limited to the order Cladophorales. These results demonstrate that chloroplast mRNA polyuridylylation is not the sole provenance of photosynthetic alveolates and may have convergently evolved in two distinct photosynthetic lineages.

Key index words: **chloroplast polyuridylylation; circular RNA; organelle RNA processing; reduced plastome**

***Abbreviations*: cRT-PCR, circular Reverse Transcriptase Polymerase Chain Reaction; MMLV RT, Maloney Murine Leukemia Virus Reverse Transcriptase; poly(A), polyadenylation; poly(U), polyuridylylation; RT, Reverse Transcriptase; SAP, Shrimp Alkaline Phosphatase**

The chloroplasts of land plants (embryophytes) and green algae (chlorophytes and streptophytes) arose from a single endosymbiotic event with an ancient cyanobacterium and comprise a eukaryotic lineage known as the Archaeplastida (Archibald 2015, Adl et al. 2019). These now semi-autonomous organelles have maintained a circular reduced genome (plastome) of densely packaged genes with a conserved architecture (i.e., two single copy and two inverted repeat regions; Palmer 1985, Daniell et al. 2016). Transcription of these plastomes has been extensively studied in model systems and an overview of the process was reviewed by Stern et al. (2010). Briefly, primary mRNAs are transcribed from the gene-dense plastomes as poly-cistronic primary transcripts that are endonucleolytically cleaved into individual mRNAs. The mature 5′ terminus of each is created by further exo- and endonuclease processing and are protected or marked by gene-specific stability factors. The 3′ termini are also created by a combination of endo- and exonucleases but are protected/marked by RNA secondary structures. Among embryophytes, transcripts are edited to mend deleterious genomic mutations; a phenomenon that is missing among the chlorophyte and streptophyte green algal lineages (Cahoon et al. 2017, Gallaher et al., 2018). A minority of mRNAs also require additional processing, such as intron removal or trans-splicing to assemble translatable coding regions. Finally, mRNAs are marked for degradation by the addition of a poly(A) oligonucleotide tail on their 3′ terminus.

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The plastome structures in most green algal lineages follow the general architecture found in embryophytes (Simpson and Stern 2002, Lemieux et al. 2016), but there are some extreme exceptions (Watanabe et al. 2016, Del Cortona et al. 2017). One of these is the green algal order Cladophorales within which plastomes have been reduced to individual coding regions or small numbers of genes occurring as linear single-stranded DNAs that fold into hairpin structures (Del Cortona et al. 2017). Another group of photosynthetic organisms with an equivalently reduced plastome are the peridinin dinoflagellates of the Alveolata eukaryotic lineage, which differ from the Archaeplastida in that they obtained their chloroplasts through the secondary endosymbiosis of a red alga (Janouškovec et al. 2010, Füssy and Oborník 2017, Waller and Koreny 2017). Their plastomes are made of mini-circles carrying one or a few genes required for photosynthesis (Howe et al. 2008). They also have unusual mRNA processing. The mini-circles are transcribed by way of a rolling circle mechanism (Barbrook et al. 2001, Nisbet et al. 2008, Dang and Green 2010), producing long transcripts with multiple copies of each gene that are released by endonucleases prior to translation. Most unusually, the 3′ ends of the transcripts are polyuridylylated, creating a poly(U) tail (Wang and Morse 2006, Nelson et al. 2007, Barbrook et al. 2012), which appears to mark mature translatable transcripts. These poly(U) additions have also been observed among non-peridinin dinoflagellates (Dorrell and Howe 2012, Jackson et al. 2012), and the chromerid algae (Janouškovec et al. 2010, Dorrell et al. 2014), which suggests a common evolutionary origin in the alveolate lineage. Another unusual event among these photosynthetic Alveolates is the extensive editing of their RNAs (Dang and Green 2009, Dorrell and Howe 2012, Klinger et al. 2018).

In this study, the following question was asked, does an unconventional plastome structure affect RNA processing? To address this, the 5′ and 3′ mRNA termini of the eight known chloroplast genes of *Pithophora roettleri* from the green algal order Cladophorales were screened for evidence of post-transcriptional processing. Evidence is presented for several processing events, most notably the polyuridylylation of the 3′ termini of six of the eight genes, which has not been reported for any lineage outside of the Alveolata. Other processing events include poly(A) and heteropolymeric 3′ additions, 5′ primary transcript start sites, as well as the presence of circularized RNAs.

MATERIALS AND METHODS

Pithophora roettleri *RNA extraction, circularization, amplification, and sequencing.* The *Pithophora roettleri* strain used in this study was collected from the Ogeechee River in Jewel, Georgia, October 2016, and cultured. Thallus was removed from culture medium, blotted dry with paper towels, frozen with liquid nitrogen, and ground into a powder using a ceramic mortar and pestle. The frozen powder was resuspended in the RNA lysis buffer included in Qiagen's (Germantown, MD, USA) RNeasy kit and the extraction procedure followed as outlined by the manufacturer. Total RNA was quantified using a NanoDrop Lite (Thermo-Fisher, Waltham, MA, USA) and stored at −80°C.

cDNA synthesis and subsequent PCR were completed using primers designed with Primer3 (Koressaar and Remm 2007, Untergasser et al. 2012) with the *Pithophora roettleri* chloroplast genome fragments archived in GenBank (MN049185–MN049192). Divergent primer pairs were designed that would theoretically only produce amplicons from circularized RNAs and would include the 3′ and 5′ ligation site of circularized mRNAs.

RNA termini were PCR amplified and sequenced using a circular RT-PCR protocol similar to those described in Mance et al. (2020) and Cahoon and Qureshi (2018). Briefly, linear mRNAs were circularized by mixing 2 μg of total RNA, 30 Units of T4 RNA ligase 1 (New England Biolabs, Ipswich, MA, USA), 1X T4 RNA ligation buffer, and $1U \cdot \mu L^{-1}$ of RNasin RNase inhibitor (Promega, Madison, WI, USA), and incubated at 37°C for 1 h. cDNA was produced from the artificially circularized mRNAs using half of the reaction volume from the circularization reaction as template and then adding RT buffer provided by the manufacturer, gene-specific primary reverse primers (Table S1 in the Supporting Information, primers labeled "_R1"), and water to the appropriate volume. This was heated to 65°C for 15 min, cooled, 200U MMLV RT (Promega) added, and then incubated at 42°C for 60 min. One μL of this cDNA reaction was used as template to PCR amplify the junction sites of circularized RNAs using the primary primer set (Table S1, primers labeled "_R1" and "_L1") and Phusion DNA polymerase (ThermoFisher, Waltham, MA, USA). Products were visualized using agarose gel electrophoresis. If products were present, they were diluted 10-fold and used as template for a second round of PCR amplification with the secondary primer set (Table S1, primers labeled "_R2" and "_L2"). To detect naturally circularized mRNAs, cDNA synthesis was conducted as described above but with total RNA that had not been treated with T4 RNA ligase.

3′ RACE analysis was completed following a protocol similar to Salinas-Giegé et al. (2017). The T4 RNA ligase circularization reaction was completed as described above but with the addition of a 3′ end adaptor. cDNA was synthesized as described above using a primer complementary to the 3′ adaptor. PCR was completed with the same primer used to complete cDNA synthesis and gene-specific "L" primers. Primer sequences are listed in Table S1. This experiment was completed once and with two genes, *atp*H and *psb*C.

RT-PCR confirmation of polyU additions was performed by synthesizing cDNA as described above except using 2 μg of unmodified total RNA as template and an oligo-d(A) primer. PCR amplification was carried out using oligo-d(A) as the "R" primer and gene-specific "F" primers. A second set of RT-PCR reactions were completed to serve as a positive control where a gene specific "R" primer was used for cDNA synthesis and then the same "R" primer was paired with a gene-specific "F" primer for PCR. A negative control was performed where RT was not included in the cDNA production step.

To detect the 5′ terminal ribonucleosides of primary transcripts, ligation reactions with total RNA were set up as described above but 3U of shrimp alkaline phosphatase (SAP, purchased from New England Biolabs) was used in place of T4 RNA ligase and incubated at 37°C for 30 min and then deactivated by heating to 85°C for 15 min. Immediately after the SAP treatment, T4 ligase was added to the reactions and

incubated as described above. SAP will dephosphorylate nucleoside mono-phosphate ends but not di- or tri-phosphate so only primary RNAs would retain their 5′ phosphates. T4 RNA ligase can use nucleoside mono- or di-phosphate RNAs as substrate (England et al. 1977). We reasoned that the only remaining substrate that could be circularized would be primary transcripts that had been partially dephosphorylated, that is, the gamma pyrophosphate removed but not the beta. We hypothesized that these partially dephosphorylated mRNAs would have been marked for removal and their analysis would reveal both the first nucleotide of the primary transcript and potential 3′ modifications required for mRNA degradation (Fig. S1 in the Supporting Information).

For sequencing, the secondary amplicons of all eight genes were combined for each of the three different treatments (artificially circularized, SAP + artificial circularization, or naturally circularized RNAs) and deep sequenced using Genewiz's (South Plainfield, NJ, USA) Illumina MiSeq Amplicon-EZ service (San Diego, CA, USA). The production and sequencing of amplicons from the three conditions were completed twice from independently isolated total RNA.

Sequence analysis. Sequences from the eight genes were sorted using the bioinformatics program, Geneious (BioMatters Ltd., Auckland, New Zealand). Sequences matching each gene were identified by aligning them to their respective coding regions using the Map to Reference function (sequences were untrimmed, minimum mapping quality of 30, and maximum mismatches per read 20%, alignment with a single pass —fast/read mapping). Aligned sequences were visually inspected for the presence of 3′ poly-nucleotide additions. To identify the variety of 3′ and 5′ ends, similar sequences were grouped using the de novo assembly function within Geneious Prime (untrimmed sequences, maximum mismatches per read 5%). The joined 3′ and 5′ termini were identified and delineated by matching them to genomic sequence using the National Center for Biotechnology Information's (USA) nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the "align two or more sequences" function.

To determine if non-template mRNA phenomena observed through circular RT-PCR were post-transcriptional modifications, the transcript sequences in question were aligned to genomic DNA using Geneious' Map to Reference function (no gaps allowed and 0–5% mismatches allowed per read). *Pithophora roettleri* genomic DNA was produced for a related project (B. Lor, M. Zohn, M.J. Meade, A.B. Cahoon & K.M. Manoylov, unpub. data**;** NCBI's Sequence Read Archive PRJNA591002). These data consist of 220,938,416 raw paired reads produced using Illumina MiSeq technology which, after trimming and pairing, provided 31,111,510 quality reads.

Other green algae. The cRT-PCR process was repeated on four representative species from other algal lineages to determine if this technique was exaggerating rare events that have been missed in previous studies. Additionally, it was considered possible that T4 RNA ligase could be adding nucleotides by an undiscovered or undocumented mechanism to create oligonucleotide artifacts during the ligation step. We chose to test two species from the class Chlorophyceae, *Chlamydomonas reinhardtii* (strain cc-503, plastome NC_005353) since, at the molecular level, it is arguably the most well-characterized green alga and a *Pediastrum duplex* (plastome MK895950) isolated from Central Georgia and cultured at Georgia College and State University as an example of a non-model system. *Chara vulgaris* (Class Charophyceae, plastome DQ229107) isolated from Wise, Virginia and cultured at University of Virginia's College at Wise (Cahoon et al. 2017) was analyzed as a representative of the Streptophyta lineage of green algae. Finally, a species from class Ulvophyceae, *Ulva flexuosa* (plastome NC_035823) collected from the Los Peñasquitos Lagoon, California (March 2018) and cultured at Georgia College and State University was analyzed as a member of the class Ulvophyceae. In all, 14 genes (*atp*B, *atp*F, *atp*H, *clp*P, *pet*A, *pet*B, *psa*A, *psa*B, *psb*B, *psb*C, *psb*K, *rpo*A, *rpo*C2, and *rbc*L) were chosen to represent an array of protein complexes and functions. Sequences were separated and aligned as described above for *Pithophora roettleri*.

Boodlea composita was analyzed as a representative of Cladophorales by utilizing sequence data archived by Del Cortona et al. (2017)—total RNA deposited into NCBI's Sequence Read Archive (SRR5500907) and plastome sequences deposited into GenBank—*atp*A (MG257797), *atp*B (MG257798), *atp*H (MG257800), *pet*A (MG257805), *pet*B (MG257809), *psa*B (MG257812), *psa*C (MG257813), *psb*A (MG257815), *psb*B (MG257816), *psb*C (MG257817), *psb*D (MG257818), *psb*L (MG257823), *psb*T (MG257825), and *rbc*L (MG257828). Oligonucleotide additions were detected using Geneious. Coding regions were extracted from each of the plastome files and used as template and total RNA aligned to genomic DNA using Geneious' Map to Reference function (no gaps allowed and 5–10% mismatches allowed per read). Alignments were then visually inspected for the presence of polynucleotide additions.

RESULTS

Modifications to the plastid mRNA 3′ termini. The eight available chloroplast encoded genes of *Pithophora roettleri* (*pet*A, *pet*B, *atp*B, *atp*H, *psa*B, *psb*B, *psb*C, and *rbc*L) were screened for mRNA end processing events using circular RT-PCR. Six were found to have post-transcriptional uridine additions on their 3′ termini consistent with untemplated homopolymeric polyuridylylation (Fig. 1). These results were confirmed using oligo-d(A) RT-PCR for a subset of these genes (Fig. S2 in the Supporting Information) and by 3′ RACE analysis of *atp*H and *psb*C. These additions occurred directly following the stop codon with the exception of *psb*B and *atp*H where it occurred one nucleoside downstream of the stop codon. For three of the transcripts (*atp*H, *psb*B, and *rbc*L), polyuridylylation was the only non-template polynucleotide addition detected (Fig. 1A). A second phenomenon, homopolymeric polyuridylylation followed by homopolymeric polyadenylation, poly (U)+poly(A), was detected on *pet*B, *psa*B, and *atp*B mRNAs (Fig. 1,B–D). This variant was found nearly exclusively among transcripts that had been treated with Shrimp Alkaline Phosphatase (SAP) prior to artificial circularization with T4 RNA ligase (Table 1). As described in Materials and Methods and diagrammed in Figure S1, SAP treatment was used to define the transcriptional start sites but it also revealed putative 3′ end modifications associated with mRNAs marked for degradation.

Two transcripts were exclusively polyadenylated on their 3′ termini, *pet*B and *psa*B (Fig. 1). In *pet*B, poly(A) tails were added four nucleosides downstream of the stop codon. These were only observed among transcripts that had been artificially circularized with no SAP pre-treatment (Fig. 1B, Table 1). For *psa*B, poly(A) additions were found on transcripts that had an AU-rich heteropolymeric

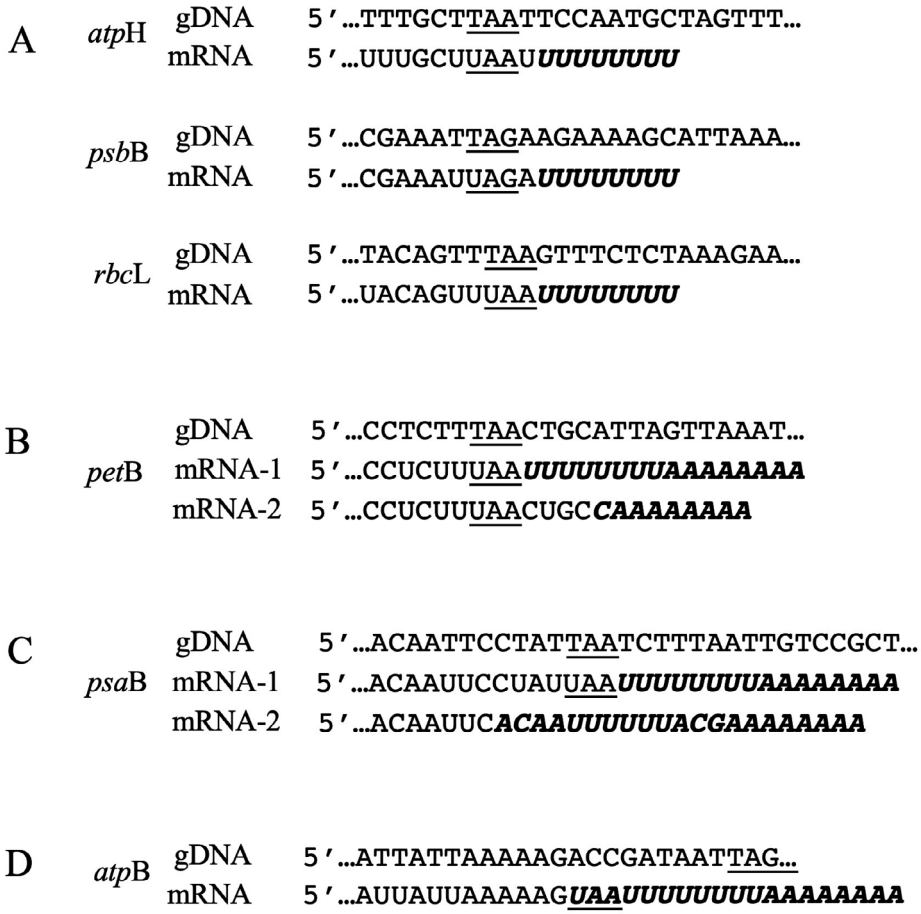

FIG. 1. Non-template oligonucleotide additions added to the 3′ termini of *Pithophora roettleri* chloroplast mRNAs. (A) Three mRNAs were polyuridylylated. (B) Two *pet*B mRNAs were detected, mRNA-1 was only found in samples pre-treated with Shrimp Alkaline Phosphatase (SAP) and had a poly(U)+poly(A) addition. mRNA-2 were detected in samples with no SAP treatment and had a poly(A) tail added 4nt downstream of the stop codon. (C) Three *psa*B mRNAs with non-template additions were characterized. Two mRNA-1 versions were detected, in non-SAP-treated samples, poly(U) was exclusively found directly following the stop codon, in samples pre-treated with SAP there was a poly(U)+poly(A) addition. mRNA-2 had a polynucleotide addition followed by poly(A) and was found in non-SAP-treated samples. (D) *atp*B mRNAs appeared to have been cleaved and a stop codon constructed de novo. These were only detected in SAP-treated samples. Abbreviations: gDNA, genomic DNA; mRNA, messenger RNA, underlined text are the predicted stop codons.

TABLE 1. Oligonucleotide additions detected on the chloroplast mRNAs of *Pithophora roettleri*.

	polyU		polyA		polyU + polyA	
Gene	Linear$^+$	Circular	Linear$^+$	Circular	Linear$^+$	Circular
*atp*B	n/d	n/d	n/d	n/d	72.60%[a]	n/d
*atp*H	92.3%[a,b]	0.0009%	n/d	n/d	n/d	n/d
*pet*A	n/d	n/d	n/d	n/d	n/d	n/d
*pet*B	0.83%	0.0004%	98%[b]	56.48%	93.77%[a]	n/d
*psa*B	78.23%[a]	0.0001%	83.91%[b]	92.71	21.08%[a]	n/d
*psb*B	67.58%[a,b]	n/d	n/d	n/d	n/d	n/d
*psb*C	n/d	n/d	n/d	n/d	n/d	n/d
*rbc*L	94.84%[a,b]	3.70%	n/d	n/d	n/d	n/d

linear$^+$, mRNAs treated with T4 RNA ligase that would contain artificially circularized mRNAs as well as an unknown amount of naturally circularized mRNAs.

Circular, naturally circularized mRNAs.

[a]This addition was primarily observed in samples treated with alkaline phosphatase prior to ligation.

[b]This addition was primarily observed in samples that were ligated without alkaline phosphatase pre-treatment.

untemplated addition on the 3′ terminus and no discernable stop codon (Fig. 1C, *psa*B mRNA-2). Genomic DNA was screened for the modifications seen in *psa*B mRNA-2 and no evidence of this AU-rich region was found, suggesting it is a non-template mRNA addition. This transcript variant was nearly exclusively found (83.91%) among transcripts that had been artificially circularized with no SAP pre-treatment (Table 1).

A second non-template modification was found in *atp*B (Fig. 1D). In this case, a stop codon was constructed de novo followed by a poly(U)+poly(A) addition. As with *psa*B, this stop codon could not be found in genomic DNA, suggesting it was a post-transcriptional addition. This phenomenon was only detected among transcripts that had been treated with SAP prior to T4 RNA ligation.

Plastid mRNA 5′ termini. For most of the genes, more than one 5′ terminus was observed in the circular RT-PCR data and it was unclear which represented the transcriptional start site and which were due to nucleolytic processing or degradation. To test this, RNA was treated with SAP prior to circular RT-PCR to dephosphorylate 5′ termini with a mononucleoside pyrophosphate, rendering them incapable of circularization. The ribonucleoside triphosphate added by RNA polymerase to initiate the primary transcript and any diphosphate modified versions would remain. The diphosphate modified transcripts could be circularized by T4 RNA ligase and should signify the modified 5′ ends of primary transcripts. This strategy reduced and/or eliminated the variety of 5′ termini for each transcript and revealed a single 5′ terminus, which was presumably the first ribonucleoside added at transcription initiation (Fig. 2). The exception was *pet*A, which had a much longer predominant 5′ UTR after SAP treatment than from non-SAP-treated transcripts, suggesting a 5′ terminus may have been created post-transcriptionally by endonucleolytic cleavage.

Nucleotides upstream of the theoretical transcriptional initiation sites were aligned to see if there was an identifiable promoter sequence. The first 25 nucleotides were AT rich but there was no canonical "TATA" box. An "AATTATTT" motif was predicted by a Logo plot (Fig. S3 in the Supporting Information).

Naturally circularized plastid mRNAs. The use of circular RT-PCR to define 3′ and 5′ termini of mRNAs assumes that all the circularized transcripts were created artificially by T4 RNA ligase. Naturally occurring circular RNAs, however, have been detected in both chloroplasts and mitochondria so circular RT-PCR was repeated without pre-treating the RNA with T4 ligase to see if any of the joined termini detected in previous experiments had occurred naturally.

Naturally circularized RNAs (circRNA) were detected from all eight transcripts (Fig. 3). For seven, the circRNAs were a heterogeneous mixture made from various 5′ and 3′ termini of differing abundance. For example, four different *rbc*L circRNAs were detected (Fig. 3A). All contained a full-length *rbc*L coding region but varied in the lengths of the 3′ and 5′ UTRs that were apparently ligated. The most common one (49.7% of the reads) was made when the terminus of the longest detected 5′ UTR (194 nt) was ligated to the terminus of a 415 nt 3′UTR to make a 3,441 nt circRNA. The largest circRNA (3,471 nt), and second most common (35.3% of the reads) was produced from a 3′ UTR with 30 nt removed from the 3′ terminus. The third most common (11.1%) was 1,926 nt while the least abundant (3.7%) and smallest (1,466 + poly(U)) was made when a polyuridylylated transcript was ligated to the 49 nt 5′ UTR. Three other transcripts (*pet*B, *atp*H, and *psa*B) had circRNA patterns similar to *rbc*L (i.e., multiple circRNAs each with a full-length coding region and the inclusion of a polynucleotide addition in at least one of the circRNA species; Fig. 3A). An exception was *psa*B where a single circRNA was detected. The circRNAs of the remaining four transcripts (*pet*A, *psb*B, *psb*C, and *atp*B) had truncated coding regions, suggesting they are incapable of supporting translation. The shortened coding regions in *pet*A, *psb*B, and *psb*C were all in the 3′ ends (Fig. 3B). Some of the *atp*B transcript ligation sites were unique among the eight because the ligations occurred between two repeated sequences (Fig. 3C, motifs 1 and 2).

The longest naturally ligated 5′ terminus matched the predicted primary transcript initiation sites for three genes (*psa*B, *atp*H, and *psb*B). For three others, the longest 5′ terminus was a short distance upstream of the predicted start site: 14nt upstream for *pet*A, 8nt for *atp*B, and 4nt for *psb*B.

3′ polyuridylylation was only detected among green algae in the Cladophorales. 14 mRNAs (*atp*B, *atp*F, *atp*H, *clp*P, *pet*A, *pet*B, *psa*A, *psa*B, *psb*B, *psb*C, *psb*K, *rpo*A, *rpo*C2, and *rbc*L) from four green algal species (*Chlamydomonas reinhardtii*, *Chara vulgaris*, *Pediastrum duplex*, and *Ulva flexuosa*) were analyzed using the same cRT-PCR methodology used for *Pithophora roettleri* and analyzable data were produced for most of them (Table 2). In addition, archived data for the 14 *Boodlea composita* chloroplast genes were analyzed to screen for evidence of oligonucleoside additions. For *C. reinhardtii*, *C. vulgaris*, *P. duplex*, and *U. flexuosa*, no oligonucleoside additions were detected. This demonstrates that poly(A) and poly(U) additions are not a widespread but overlooked phenomenon among green algae. It also demonstrates that the cRT-PCR technique did not add spurious nucleotides prior to sequencing. Poly(A) and poly (U) additions were found associated with *atp*A and *psa*C from the archived *B. composita* RNA. This suggests that these oligonucleoside additions are limited to a single green algal lineage.

DISCUSSION

Chloroplast oligonucleoside additions. The addition of oligonucleosides to the 3′ termini of mRNAs is a

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                                    5′– AAACAAUA…   petB    5 nt
                                 5′– AAAUAAAAAGAACAUG…   atpB   13 nt
                               5′– AAAGAAAUAACUAUCAUU…   psaB   14 nt
                          5′– AGUAGGAUAAUCUAUUGAAUCCUUAUUACUAUG…   atpH   30 nt
                     5′– AUUAUUCUUAAUCACAUUAAUCUGAUCAUUAUUUAUUUACUAAUG…   psbB   42 nt
                5′– CGGGCUUAAUUGCCCACUUUUACAUUAGGAGCUGUUUAUUUAUCUCUUUCAUG…   rbcL   50 nt
           5′– UCAUUAUUUCUUGGAAUCAUUUAAGAGGUAUCAGACUUUUGUGGGCCGGUAAUG…   psbC   51 nt
      5′– UAGUUUUAAUUAUUGAACCAAUAACAUUAGGUAUUGUUUUAGGCCUCUUCACUAU
          UUCCGUAAUUGGUCUAAUUAUUUCGGCAAUUGGUCAGUUUGUUCUUACUACUAAGAUU…   petA   113 nt
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FIG. 2. 5′ primary transcript termini of *Pithophora roettleri* chloroplast mRNAs. mRNAs were pre-treated with shrimp alkaline phosphatase prior to ligation with T4 RNA ligase to determine the initial nucleoside addition by RNA polymerase. Underlined nucleotides represent the predicted start codons for each gene. The number to the right of each gene name is the length of the 5′ untranslated region of each primary transcript.

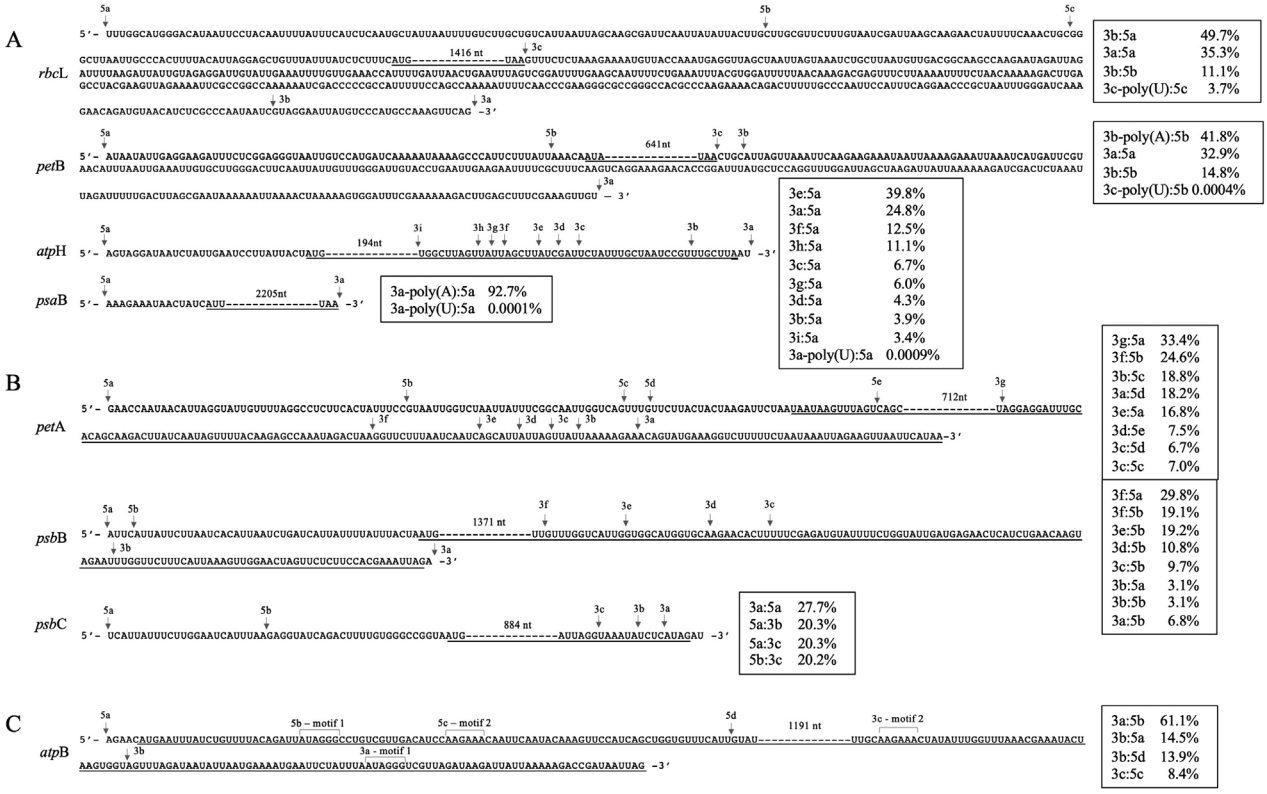

FIG. 3. Naturally circularized RNAs (circRNAs) in the chloroplasts of *Pithophora roettleri*. The 5′ and 3′ termini (arrowheads) that were naturally joined to produce circRNAs were mapped for each gene. To the right of each mRNA is a list the predominant circRNAs and their relative abundance compared to all the circRNAs detected for that gene. Coding regions are underlined and dashes represent nucleosides removed to conserve space.

commonly accepted phenomenon (i.e., polyadenylation of nuclear transcripts). Among organelles, there is a wide variety of observed additions. In the mitochondria of green algae, oligonucleoside additions are prevalent and include poly(A), poly(U), and poly(C) with the choice of nucleoside presumably playing a role in either mRNA maturation or degradation (Zimmer et al. 2009, Salinas-Giegé et al. 2017, Cahoon and Qureshi 2018, Gallaher et al. 2018). In the chloroplasts of algae and angiosperms, these additions appear to be limited to poly (A) where it acts as a mRNA degradation signal and can only be found on an "extreme minority" of transcripts (Lisitsky et al. 1996, Komine et al. 2000, 2002, Gallaher et al 2018). An exception to this paucity of chloroplast oligonucleoside additions is the widespread addition of poly(U) tails on the chloroplast mRNAs of photosynthetic Alveolates where it

TABLE 2. Oligonucleotide additions on chloroplast mRNAs as detected using cRT-PCR.

Gene	*Pithophora roettleri*	*Boodlea composita*[a]	*Ulva flexuosa*	*Chlamydomonas reinhardtii*	*Pediastrum duplex*	*Chara vulgaris*
*atp*A		+				n/d
*atp*B	+	n/d		n/d		n/d
*atp*F				n/d	n/d	n/d
*atp*H	+	n/d	n/d	n/d	n/d	n/d
*clp*P				n/d	n/d	n/d
*pet*A	n/d	n/d	n/d	n/d		n/d
*pet*B	+	n/d	n/d	n/d		n/d
*psa*A			n/d	n/d	n/d	n/d
*psa*B	+	n/d		n/d	n/d	n/d
*psa*C		+				
*psb*A		n/d	n/d			
*psb*B	+	n/d	n/d	n/d	n/d	n/d
*psb*C	n/d	n/d	n/d	n/d		n/d
*psb*D		n/d	n/d			n/d
*psb*K				n/d	n/d	
*psb*L		n/d				
*psb*T		n/d				
*rpo*A				n/d		n/d
*rpo*C2			n/d	n/d	n/d	n/d
*rbc*L	+	n/d		n/d	n/d	n/d

+, 3′ oligonucleotide additions were detected on this gene's mRNA.
n/d, No oligonucleotide additions were detected.
blank, No data were collected for this gene.
[a]These data were derived from archived sequences rather than cRT-PCR.

has been presumed to uniquely occur (Wang and Morse 2006, Janouškovec et al. 2010, Dorrell et al. 2014). The photosynthetic alveolate lineage has a secondary endosymbiont of red algal origin and has given rise to the chromerid algae, peridinin dinoflagellates, and apicomplexans (Janouškovec et al. 2010, Füssy and Oborník 2017, Waller and Koreny 2017). Among these, poly(U) tails are primarily added to transcripts encoding proteins used in the photosynthetic electron transport chain. Interestingly, plastid-carrying Alveolates that lack photosynthesis, apicomplexans, lack polyuridylylation (Dorrell et al. 2014, Nisbet and McKenzie 2016).

The poly(U) additions we found on *Pithophora roettleri* occurred directly following, or within one nucleoside of the predicted stop codons with little variation. Transcripts with longer 3′ UTRs (e.g., *rbc*L and *pet*B) were detected, which suggests that although the transcripts are mono-cistronic they are still endonucleolytically cleaved on their 3′ ends prior to polyuridylylation. The pervasiveness of poly (U) among the analyzed reads demonstrates that transcripts without this addition were a minority and suggests that uridylylated transcripts support translation rather than marking them for degradation. This is consistent with observations from the photosynthetic Alveolate lineages. In dinoflagellates, poly(U) additions appear on mature and translationally competent mRNAs (Barbrook et al. 2012, Dorrell et al. 2014, 2016, Richardson et al. 2014). In chromerids, the poly(U) tail is added after endonucleolytic processing of the 3′ UTR and the addition is associated with high steady-state levels of transcripts related to photosynthesis (Janouškovec et al. 2013, Dorrell et al. 2014).

Unlike polyuridylylation, polyadenylation of chloroplast transcripts is a presumably universal phenomenon among the Archaeplastida where it acts as a degradation signal (Lisitsky et al. 1996, Komine et al. 2000, 2002). We found poly(A) additions associated with three *Pithophora* transcripts, *atp*B, *pet*B, and *psa*B, and its detection was determined by the experimental approach, namely whether or not the transcripts were treated with alkaline phosphatase prior to circularization. In *pet*B, 98% of the circular RT-PCR reads of non-SAP-treated transcripts had a poly(A) addition 4 nt downstream of the stop codon, suggesting the transcript had been endonucleolytically cleaved and then polyadenylated. When the same samples were treated with SAP, 93.77% of the reads were polyuridylylated first and then polyadenylated. A similar poly(U)+poly(A) phenomenon was observed for *atp*B and *psa*B. Since SAP treatment would have preferentially selected for the ligation of 5′ termini with diphosphate ends, the appearance of specific 3′ processing events in these assays suggests there is a correlation between processing events at the 5′ and 3′ ends. This is most likely related to an mRNA degradation pathway. In *Escherichia coli*, the conversion of a 5′ nucleoside triphosphate to a di- or mono-phosphate initiates mRNA degradation by way of RNase E endonucleolytic cleavage (Celesnik et al. 2007, Deana et al. 2008, Luciano et al. 2017, 2018) while in *Bacillus subtilis* it triggers exonucleolytic degradation by RNase J (Mathy et al. 2007, Richards et al. 2011). If an equivalent mRNA removal

pathway exists in chloroplasts, then the 5′ ends with a nucleoside diphosphate have been modified, marking them for degradation. The co-occurrence of a poly(A) tail on the 3′ end following a poly(U) addition suggests that polyadenylation also marks the mRNA for removal. For example, the majority of *psa*B transcripts treated with SAP exclusively had a poly(U) addition while a minority had poly(U)+poly(A). This suggests that the dephosphorylation event preceded the addition of the poly(A). The absence of poly(U)+poly(A) in non-SAP-treated transcripts suggests it only occurs with a modified 5′ terminus and that both are associated with mRNA degradation. This is consistent with an observation from the green alga *Chlamydomonas reinhardtii* where transcripts are polyadenylated as a degradation signal (Komine et al. 2000).

Two heteropolymeric additions were also found among the 3′ UTRs. The first example is a non-template UAA stop codon added to *atp*B transcripts. This stop codon was only found among SAP-treated transcripts where it was followed by a poly(U) + poly(A) addition. The construction of a stop codon has been observed in human mitochondria where coding regions are separated by extremely short intergenic spaces. Endonucleolytic cleavage leaves a single uracil in the stop codon position and polyadenylation completes it (Anderson et al. 1981, Nagaike et al. 2005). The *Pithophora roettleri atpB* case differs from human mitochondria in that endonucleolytic cleavage to remove adjacent coding regions is presumably unnecessary. Also, the stop codon appears to have been created de novo rather than needing to be completed by an oligonucleoside addition. The second example was the addition of a 13nt A and U rich addition on truncated *psaB* transcripts. Heteropolymeric additions have been previously described on chloroplast mRNAs of higher plants where they act as degradation signals (Lisitsky et al. 1996). The presence of a homopolymeric poly(A) addition downstream of the heteropolymeric addition on the *P. roettleri psa*B suggests that these transcripts are also marked for degradation.

Polyuridylylation is limited to the Cladophorales. poly (U) and poly(A) additions were found on the 3′ termini of mRNAs in the chloroplasts of the green alga *Pithophora roettleri*. Archived data from *Boodlea composita* were also analyzed and similar additions were found. Both species are in the order Cladophorales and class Ulvophyceae, which is grouped with the classes Trebouxiophyceae and Chlorophyceae to form a clade (TUC) containing the majority of green chlorophyte algae (Leliaert et al. 2012). We screened three other chlorophyte algae from the TUC clade and one streptophyte alga using the same cRT-PCR methodology and found no evidence of oligonucleoside additions. This suggests that chloroplast poly(U) additions may uniquely occur within the Cladophorales green algal lineage.

Species within the Cladophorales have non-canonical chloroplast genomes where one or a few genes are carried on small, linear, single-stranded DNAs with extensively folded hairpin structures (Del Cortona et al. 2017). Intriguingly, these fragments almost exclusively carry genes required for photosynthesis, similar to the mini-circle genomes of peridinin-containing dinoflagellates. It is possible that chloroplast poly(U) additions convergently evolved in this algal lineage to accommodate their reduced chloroplast genomes. We hypothesize that when a plastome is fragmented and large portions transferred to the nuclear genome, some genes will co-occur in both organelles. Perhaps the poly(U) addition distinguishes chloroplast mRNAs from those produced in the nucleus.

RNA circularization. We found what appears to be circularized RNAs made from the eight known *Pithophora roettleri* chloroplast genes. Covalently closed circular RNAs are common in all branches of life but their prevalence was under-appreciated until the widespread adoption of next-generation sequencing which revealed their extensive occurrence among the nuclear genes of animals and plants (reviewed in Lasda and Parker 2014, Vicens and Westhof 2014, Lai et al. 2018, Wilusz 2018). Among organelles, chloroplast circRNAs have only been reported from *Arabidopsis thaliana* (Sun et al. 2016, Liu et al. 2019) but from the mitochondria of multiple organisms, including yeast, a green alga, barley, *Arabidopsis thaliana*, and human cell lines (Amberg et al. 1980, Darbani et al. 2016, Sun et al. 2016, Cahoon and Qureshi 2018, Zhang et al. 2019, Mance et al. 2020).

In *Pithophora roettleri* chloroplasts, circRNAs were comprised of heterogeneous mixtures made from differing 3′ and 5′ termini. For most of these genes, it is unknown if these termini resulted from endonucleolytic cleavage or random shearing, but for one, *atp*B, there were intriguing short (6 nt) repeated sequences at two of the junction sites. In mammalian nuclear circRNAs, short repeat sequences are necessary for the back-splicing mechanism that circularizes various exon combinations (Jeck et al. 2013, Liang and Wilusz 2014) and in human mitochondria, junction sites appear to have a nucleoside bias (Mance et al. 2020). The circularization mechanism used in organelles is unknown but the presence of conserved repeat sequences at junction sites suggests that circularization factors target and produce specific circRNAs, which in turn suggests intentional functionality for this class of RNA as well as the possibility it could be related to the mechanism found in the nucleus.

For four of the *Pithophora roettleri* genes (*pet*A, *psb*B, *psb*C, and *atp*B), only circRNAs from fragments of the coding regions were detected. These are similar to the circRNAs reported from *Arabidopsis*

chloroplasts, barley mitochondria, and human mitochondria (Darbani et al. 2016, Sun et al. 2016, Mance et al. 2020). The function of circRNAs in organelles is unknown but for nuclear genes a number of mechanisms have been proposed including miRNA sequestration (Zheng et al. 2016), protein interaction (Du et al. 2016), and transcriptional and post-transcriptional gene regulation (Salzman et al 2012, Huang et al. 2017). These mechanisms are congruent with the circRNAs described in this study made from fragmented coding regions that would be incompatible with translation. In *Arabidopsis* chloroplasts, the steady-state levels of specific circRNA gene fragments have been shown to differ in etiolated versus de-etiolated seedlings (Liu et al. 2019) and in barley mitochondria circRNAs fluctuate in relation to micronutrient levels (Darbani et al. 2016), suggesting they play some role in homeostasis. The exact roles they play have yet to be determined.

For the remaining *Pithophora roettleri* genes (*rbc*L, *pet*B, *atp*H, and *psa*B), full-length coding regions were circularized, raising the possibility they may be translated. In some studies, circRNAs have been found to co-sediment with ribosomes suggesting they are compatible with translation (Cahoon and Qureshi 2018, Ragan et al 2019) and protein production has been confirmed from a mouse nuclear circRNA via Western blots and mass spectrometry (Mo et al. 2019). Conversely, we observed that an exceedingly small portion of the natural circRNAs from *rbc*L, *pet*B, *atp*H, and *psa*B were ligated after a poly(U) addition. If poly(U) addition is necessary to create a translationally competent mRNA, then circularization may be incompatible with translation in *P. roettleri* chloroplasts. Further experimentation is needed to determine if chloroplast circRNAs are indeed translated.

For the genes *pet*B and *psa*B, exclusive oligoadenylated tails made up the majority of the naturally circularized mRNAs. This suggests that the addition of an exclusive poly(A) tail and RNA circularization are complementary RNA processing steps. We entertained the idea that a poly(A) addition serves as a signal for circularization but since no poly(U)+poly(A) additions were observed among naturally circularized mRNAs we do not believe this to be plausible.

A caveat with the *Pithophora roettleri* data is that the RNA was not pre-treated with RNAse-R to remove linear RNAs prior to the production of cDNA, as in other published studies of organellar circular RNAs (Cahoon and Qureshi 2018, Mance et al. 2020). This creates the possibility that some or all of the products could have been RNA chimeras.

CONCLUSIONS

In this study, we characterized the chloroplast mRNAs of *Pithophora roettleri*, a green alga in the order Cladophorales and found evidence of 3′ and 5′ endonucleolytic cleavage, the addition of poly(U), poly(A), poly(U)+poly(A), and heteropolymeric tails to the 3′ termini, and transcript circularization. To the best of our knowledge, this is the first report of polyuridylylation in a green algal chloroplast and the first report of chloroplast transcript circularization outside of angiosperms. We were unable to find oligonucleoside additions in four other green algal species. This provides evidence that this phenomenon may be limited to the class Cladophorales and we speculate it may be related to their highly reduced chloroplast genomes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Diagrammatic representation of the reasoning behind the Shrimp Alkaline Phosphatase treatments and subsequent interpretation of results.

Figure S2. Reverse-Transcriptase PCR amplification of portions of five *Pithophora roettleri* mRNAs. A. A polyA primer was used to produce cDNA from mRNAs with a polyU tail. The cDNAs were then PCR amplified using a gene specific left primer and polyA. Amplicons of the predicted size are highlighted with an asterisk. In the cases of *psa*B and *rbc*L amplicons other than the predicted one were produced but their identities are unknown. Primer sequences are described in Table S1. The *cox*I mitochondrial mRNA (GenBank MN049192) was included as a negative control since it has no polyU addition (Proulex, Meade, and Cahoon, unpublished data) and occurs in a different organelle. B. Gene specific right primers were used to produce cDNAs from each mRNA. The same primer was paired with a gene specific left primer for PCR amplification. C. The reactions shown in Figure S2A were repeated with no Reverse Transcriptase step.

Figure S3. Logo plot of the 25 nucleotides upstream of the 5′ primary transcript termini from the 8 chloroplast genes of *Pithophora roettleri*. The plot was generated using, https://weblogo.berkeley.edu/logo.cgi

Table S1. Synthetic oligos used in this study.