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Tracking the elusive 5′ exonuclease activity of *Chlamydomonas reinhardtii* RNase J

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Abstract

Key message ***Chlamydomonas*** **RNase J is the first member of this enzyme family that has endo- but no intrinsic 5′ exoribonucleolytic activity. This questions its proposed role in chloroplast mRNA maturation.**

Abstract RNA maturation and stability in the chloroplast are controlled by nuclear-encoded ribonucleases and RNA binding proteins. Notably, mRNA 5′ end maturation is thought to be achieved by the combined action of a 5′ exoribonuclease and specific pentatricopeptide repeat proteins (PPR) that block the progression of the nuclease. In *Arabidopsis* the 5′ exo- and endoribonuclease RNase J has been implicated in this process. Here, we verified the chloroplast localization of the orthologous *Chlamydomonas* (Cr) RNase J and studied its activity, both in vitro and in vivo in a heterologous *B. subtilis* system. Our data show that Cr RNase J has endo- but no significant intrinsic 5′ exonuclease activity that would be compatible with its proposed role in mRNA maturation. This is the first example of an RNase J ortholog that does not possess a 5′ exonuclease activity. A yeast two-hybrid screen revealed a number of potential interaction partners but three of the most promising candidates tested, failed to induce the latent exonuclease activity of Cr RNase J. We still favor the hypothesis that Cr RNase J plays an important role in RNA metabolism, but our findings suggest that it rather acts as an endoribonuclease in the chloroplast.

Keywords RNase J · *Chlamydomonas reinhardtii* · Endoribonuclease · Exoribonuclease · RNA metabolism

Introduction

mRNA maturation and degradation are fundamental processes that make important contributions to the control of gene expression. RNase J is a key ribonuclease involved in these processes. Discovered in *B. subtilis* as an endoribonuclease with RNase E-like cleavage specificity (Even et al. 2005), RNase J1 is also the only known bacterial 5′ exoribonuclease (Mathy et al. 2007). Both activities are catalyzed by the same active site but the exonuclease activity of *B. subtilis* RNase J1 requires a monophosphorylated 5′ end to dock into a specific mononucleotide-binding pocket (Li et al. 2008). RNase J is widely distributed in bacteria and when it has been investigated, all RNase J proteins have shown this dual activity (Laalami et al. 2014). However, in contrast to the robust 5′ exonuclease activity of *B. subtilis* RNase J1, the paralogous RNase J2 has a much weaker exonuclease activity. Interestingly, the archaeal RNase J orthologs appear to have only a 5′ exonuclease activity (Clouet-d'Orval et al. 2010). This suggests that dual endo- and exoribonucleolytic activity is not necessarily a general property of RNase J homologs (Even et al. 2005; Mathy et al. 2010).

In addition to their frequent occurrence in prokaryotes, orthologs of RNase J are ubiquitous in plants, where the enzyme is targeted to the chloroplast (Sharwood et al. 2011; this work). This semi-autonomous organelle is derived from a cyanobacterial ancestor. Chloroplast RNA metabolism employs mechanisms reflecting the dual origins of its

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genetic make-up. RNA stability, editing and splicing depend heavily on a number of RNA-binding proteins that do not occur in bacteria. However, the major ribonucleases involved in these processes are of prokaryotic origin (Barkan 2011; Stern et al. 2010). *Arabidopsis* RNase J has been described as a dual activity ribonuclease similar to *B. subtilis* RNase J1 and shown to participate in a quality control mechanism, that eliminates transcripts thought to originate from inefficient transcription termination (Sharwood et al. 2011). The 5′ exonuclease activity has also been implicated in the maturation of chloroplast mRNA 5′ termini, with specific RNA-binding proteins acting as barriers to its activity (Luro et al. 2013). In *Chlamydomonas*, the introduction of a polyG "cage" in the 5′ UTR favored the formation of precursor mRNA extending to the polyG sequence, an observation best explained by a 5′ exonuclease activity (Loiselay et al. 2008). RNase J is a prime candidate for this role.

Chlamydomonas reinhardtii is a unicellular green alga with a diameter of about 10 micrometers, which belongs to the phylum *chlorophyta*. This group is a common inhabitant of marine and terrestrial environments (on damp soils and stagnant waters). *Chlamydomonas* has an ellipsoid shape with two apical flagellas on one side and basal chloroplast, which surrounds the nucleus. The presence of a single large chloroplast and a rapid doubling time (5–8 h) make this alga a perfect model organism, especially to study chloroplast genetics (Nickelsen and Kuck 2000).

Our initial interest in studying *Chlamydomonas* (Cr) RNase J resulted from the presence of a large C-terminal domain that is not found in prokaryotic orthologs like *B. subtilis* J1 (Bs RNase J1). To our surprise, we found that Cr RNase J has no significant intrinsic 5′ exonuclease activity that would justify its implication in the 5′ maturation processes described above. We conclude that either Cr RNase J is not the major 5′ exonuclease that it was thought to be or it requires co-factors to induce its latent activity. Despite the fact that we did not succeed in identifying this potential co-factor we still favor, and cannot exclude, the hypothesis that a yet unknown chloroplast protein might activate and thus control RNase J as an exoribonuclease.

Results

Versions of Cr RNase J

Chlamydomonas reinhardtii chloroplast RNase J is expressed from the nuclear encoded *rnj* gene. Cr RNase J has 920 amino acids, which is similar in size to RNase J from higher plants but much longer than Bs RNase J1 (555 aa), the best studied bacterial ortholog. In Fig. 1 we compare the Cr and Bs RNase J1 domain structures and illustrate the different versions of the Cr protein that were used in this study. The central part of Cr RNase J (aa 111-692) shows a similarity of 70% with full-length Bs RNase J1 and shares all signature domains and motifs that assign this enzyme to the β-CASP family of metallo-β-lactamase ribonucleases (Fig. 1 and Supp. Fig. 1). The β-CASP domain is inserted into the canonical metallo-β-lactamase domain and contributes three motifs (A, B and C) that participate in the coordination of the catalytic Zn^{2+} ions (Callebaut et al. 2002) (Supp. Fig. 1). The C-terminal sequence of 228 aa shows no significant homology with any known protein. Indeed, it is not homologous to the similarly sized C-terminal extension in *A. thaliana* (Supp. Fig. 1). The N-terminal extension (110 aa) carries a transit peptide sequence that is predicted to englobe the N-terminal 33 amino acids (ChloroP, Emanuelsson et al. 1999) or the first 21 amino acids (PredAlgo, Tardif et al. 2012). The rest of this domain has no sequence similarity to other known proteins. In *Arabidopsis* an N-terminal extension of similar length was predicted to contain a sequence of 70 aa that were shown to be sufficient to confer chloroplast targeting (Sharwood et al. 2011). In order to verify that RNase J is also targeted to the chloroplast in *Chlamydomonas* we performed a Western blot analysis of RNase J in isolated chloroplasts using an antibody raised against a peptide of Cr RNase J (Fig. 1b, gel 1). A signal corresponding in size to full-length Cr RNase J (theoretical MW: 98.4 kD) is clearly present in the chloroplast fraction (Fig. 1b, gel 1, right lane, band indicated by an arrow) as well as in a total *Chlamydomonas* cell extract (Fig. 1b, gel 1). The presence of RNase J at this position on the gel was confirmed by co-migration with full-length Cr RNase J overexpressed from a plasmid in *E. coli* and by mass spectrometry (data not shown). By contrast, RNase J was not detected by mass spectrometry at the position corresponding to the strong signal of lower molecular weight (~ 75 kD) observed in the *Chlamydomonas* extract (Fig. 1b, gel 1, upper panel, band indicated by an asterisk) indicating that it corresponds to a non-specific interaction of the antibody with a cytosolic protein.

Since the amounts of the total and chloroplast extracts loaded on the gel are based on identical chlorophyll content any protein localized in the chloroplast should be present in equal quantities in both extracts. This is clearly the case for RNase J and also cytochrome f that we used as positive control for a chloroplast localized protein (Fig. 1b, gel 1, bottom panel). By contrast, α-tubuline known to be localized in the cytoplasm is present in much higher concentration in the total cell extract. The single *Chlamydomonas* chloroplast makes up 60% of the total volume of the cell. Residual cytosolic protein like α-tubuline tested here generally contaminates chloroplast preparations from this organism and explains their detection in the Western analysis (Fig. 1b, gel 1).

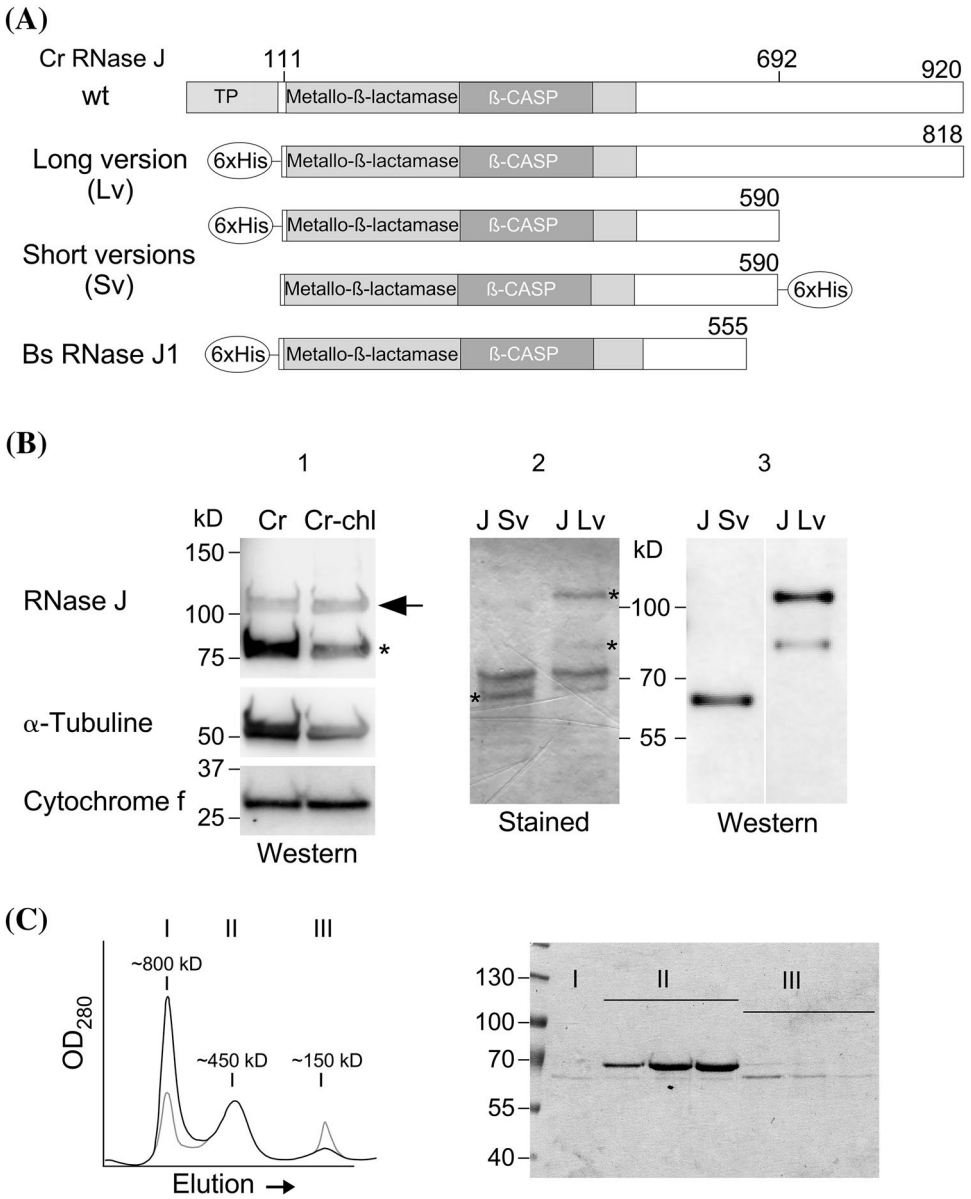

Fig. 1 Construction, purification and immunodetection of Cr RNase J variants. **a** Domain alignment of wild type Cr RNase J and constructed recombinant forms from *Chlamydomonas* (GI: 187766729) and Bs RNase J1 from *B. subtilis*. Numbers above the boxes indicate amino acid positions/length of the protein. 6×His shows the position of the His-tag in recombinant proteins. TP = approximate Transit Peptide position. **b** Gel 1: Western blot analysis (7.5% PAGE) of total *Chlamydomonas* cell extract (Cr) and chloroplast extract (Cr–chl) using antibodies against Cr RNase J, α-tubuline and cytochrome f. Aliquots from extracts containing 2 µg of chlorophyll were loaded in each lane. The arrow indicates the position of full-length Cr RNase J. The position of a cross-reacting unidentified cytosolic contaminant is marked by an asterisk. α-tubuline (50 kD) is a cytosolic marker protein, cytochrome f (32 kD) is localized in the thylakoid membrane in the chloroplast. Gels 2 and 3 (10% PAGE): short (J Sv) and long (J Lv) versions of Cr RNase J purified under native conditions (Coomassie stain, gel 2). 10 ng of purified proteins were analysed by Western blot using antibodies against Cr RNase J (gel 3). Asterisks indicate the bands corresponding to Cr RNase J as revealed by Western blot. **c** Change in gel filtration (Superdex 200) profile of Cr RNase J (Sv) purified in the presence (grey line) and absence (black line) of added RNase/DNase. Coomassie-stained SDS-PAGE gel of the three peaks fractions of the RNase/DNase treated preparation

We worked with three versions of Cr RNase J (Fig. 1a). They all lack the N-terminal 111 aa including the transit peptide and either contain the core region similar in length to the Bs J1 protein (with a N- or C-terminal His-tag) or, in addition, the specific 228 aa C-terminal extension. We preferred to work with Cr RNase J proteins missing the entire N-terminal domain (111 aa) for practical reasons (e.g. higher solubility). However, Cr RNase J lacking only the predicted

N-terminal transit peptide (33 aa) showed exactly the same in vitro activity pattern than the protein described here (data not shown).

Expression of these proteins from their native genes caused serious problems for their purification, with the formation of inclusion bodies and/or a very low amount of protein synthesized. The only way to circumvent this problem was to purify the proteins under denaturing conditions and to renature them prior to testing their activity. Since the denaturing step might lead to a definitive loss of activity we decided to purify the three versions of Cr RNase J from codon-optimized genes, which allowed the purification of soluble native proteins. All activity tests shown here have been carried out with native proteins. The purified Cr proteins were recognized by both an antibody raised against a peptide of Cr RNase J (Fig. 1b, gel 3) and also by a polyclonal antibody directed against Bs RNase J1 (data not shown) indicating a similarity of certain epitopes between the two proteins.

Cr RNase J generally purified as a soluble high molecular weight aggregate (MW ~ 800 kD, peak I in Fig. 1c) with very little protein present as a dimer (peak III in Fig. 1c). This equilibrium could be shifted significantly towards the dimer when the proteins were purified in the presence of added RNase and DNase. A large amount of a contaminating protein was co-purified but well separated by gel filtration (peak II in Fig. 1c). We identified this protein as *E. coli* bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase by mass spectrometry.

The in vitro activity of Cr RNase J was tested with all forms of purified protein, N- or C-terminal His-tag or the soluble high MW or dimer fractions. We observed no significant difference in in vitro activity based on the location of the His-tag nor the method of purification of Cr RNase J, including a gel filtration step or not. All of the assays presented here were carried out with N-terminally His-tagged Cr RNase J proteins isolated under non-denaturing conditions.

In vitro activity of Cr RNase J

To explore the catalytic properties of Cr RNase J, the purified short and long versions of the protein were incubated with 5′ tri- and 5′ mono-phosphorylated *thrS* leader transcripts of *B. subtilis*, the same transcript that we had previously used to analyze Bs RNase J1 activity (Li et al. 2008). This transcript carries a well-known RNase J endonucleolytic cleavage site (Even et al. 2005). On the 5′ triphosphorylated (PPP) RNA, the short and long versions of Cr RNase J showed a level of endonucleolytic activity comparable to that of Bs RNase J1 (~ 30–50%, Fig. 2a, left panel), as estimated from the amount of the upstream cleavage product (~ 243 nt). We could never detect the short ~ 37 nt 3′ cleavage product, most likely due to the low number of U residues contained in this short fragment. Therefore, we carried out the same reaction with a longer 351 nt *thrS* leader transcript. The reaction mixture contained both the prematurely terminated 280 nt transcript and the 351 nt readthrough mRNA that contains an additional 71 nt on the 3′ end. This allowed the simultaneous detection of both cleavage products, the ~ 243 nt upstream fragment (identical for both *thrS* transcripts) and the expected 3′ cleavage product of the longer transcript (~ 108 bases, marked by a black triangle in Fig. 2a, right panel).

On the 5′ end-labeled monophosphorylated (P) substrate Bs RNase J1 shows, as expected, a strong 5′ exonucleolytic activity producing large amounts of mononucleotides. This is not the case for both the long and short form of Cr RNase J; almost no 5′ exonucleolytic activity was detected, and instead we observed primarily a pentameric cleavage product (Fig. 2b) similar to cleavages close to the 5′ end that we have previously observed with other RNase J proteins and that we interpret as a "sliding endonuclease" activity (Taverniti et al. 2011) (see "Discussion").

In order to determine whether Cr RNase J really has no significant 5′ exonucleolytic activity we carried out a new series of in vitro assays using unrelated 5′ end-labeled monophosphorylated oligoribonucleotides (24mers). These RNAs only differed in their first nucleotide (A, C, G or U) and were unable to form significant secondary structures ($\Delta G < 1.3$ kcal/mol). Again, Bs RNase J1 efficiently hydrolyzed the RNA to mononucleotides, but no 5′ exonucleolytic degradation was observed, neither with the long nor the short version of Cr RNase J (Fig. 2c). Moreover, the reaction patterns for all substrates were very similar showing that the identity of the first nucleotide plays no role for the initiation of the Bs RNase J1 5′ exonucleolytic activity nor can it explain the absence of the exonuclease activity of Cr RNase J.

In an attempt to activate any latent exonucleolytic activity, we tested the influence of divalent ions and pH on Cr RNase J activity. Bs RNase J1 had equivalent 5′ exonucleolytic activity in the presence of Mg^{2+} or Mn^{2+} ions, the presence of Mn^{2+} did not induce or stimulate the potential 5′ exonuclease activity of Cr RNase J (Fig. 3a) as was observed for some bacterial RNase J enzymes (Hausmann et al. 2017; Zhao et al. 2015). Addition of 1 mM Zn^{2+}, generally found in the catalytic center of RNase J proteins, also had no positive effect (data not shown). Varying the pH of the reaction buffer did not produce any increase in the extremely low 5′ exonuclease activity of Cr RNase J. In contrast, Bs RNase J1 was fully active in the range from pH 6 to 9 (Fig. 3b).

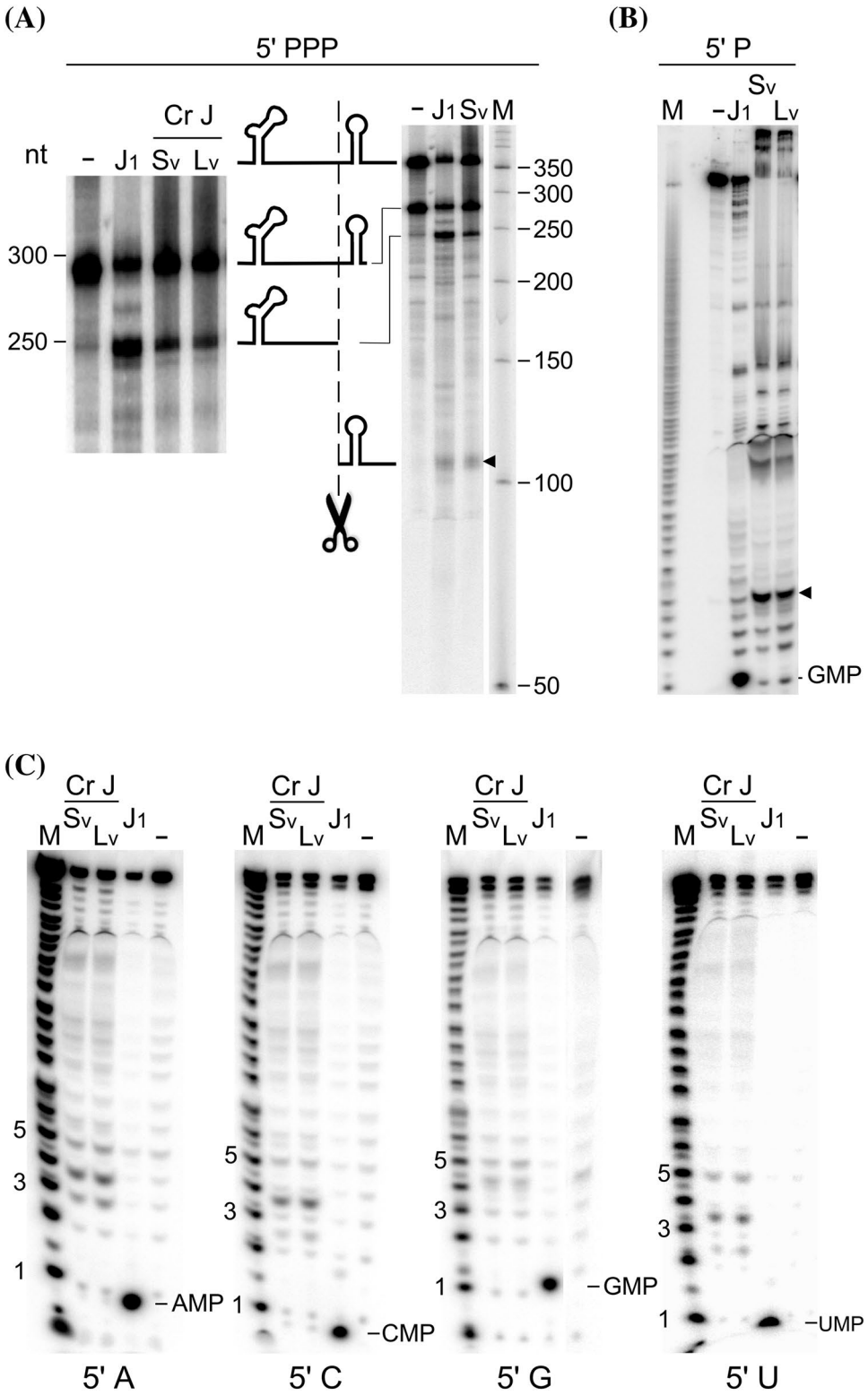

Fig. 2 Cr RNase J has endo- but no significant 5′ exonuclease activity in vitro. **a** 5′ PPP ^{32}P-UTP continuously labeled *thrS* transcripts were incubated with Bs RNase J1 (J1) and the short (Sv) and long (Lv) versions of Cr RNase J. Left panel: cleavage of the 280 nt *thrS* leader mRNA (ending with the terminator on the 3′ end) upstream of the terminator structure. The ~243 nt upstream cleavage product is indicated. Right panel: cleavage reactions with Bs RNase J1 and the short version of Cr RNase J (Sv) were carried out on a mixture of both the 280 nt *thrS* leader mRNA and a longer 351 nt read-through *thrS* transcript (+71 nt at the 3′ end with respect to the 280 nt *thrS* transcript). Reaction products were separated on a 6% urea PAGE. The expected downstream cleavage product from the 351 nt *thrS* transcript (~108 ± 1 nt) is indicated by a black triangle. M: ^{32}P-labeled 50 bp DNA ladder. **b** Cleavage of a 5′ ^{32}P end-labeled *thrS* mRNA leader transcript. Products are resolved on a 20% PAGE. M = marker generated by partial alkaline hydrolysis of the substrate. The position of GMP corresponding to the liberated 5′ terminal nucleotide is shown. A major 5 nts fragment typical for the sliding endonuclease activity of Cr RNase J is indicated by a black triangle. The full-length RNA and larger fragments are partially retained in the well due to strong binding of Cr RNase J to the RNA. **c** 5′ exonuclease assay on 5′ ^{32}P end-labeled 24mer RNA transcripts differing only in their 5′ terminal nucleotide. Numbers on the left side of the gels indicate the size of marker bands (nt). All reactions shown in Fig. 2 contained 0.3 µM Cr RNase J or Bs RNase J1. M: partial alkaline hydrolysis ladder of the substrate

In vivo activity of Cr RNase in *B. subtilis*

The in vitro assays indicated that Cr RNase J has endo- but no significant 5′ exoribonucleolytic activity. To find out whether this also holds true under in vivo conditions in a heterologous system, we decided to analyze to what extent this protein can substitute for RNases J1 (*rnjA*) and J2 (*rnjB*) in *B. subtilis*. For that purpose, we put the gene encoding the short version of Cr RNase J under control of the inducible Pxyl promoter and integrated the construct in single copy on

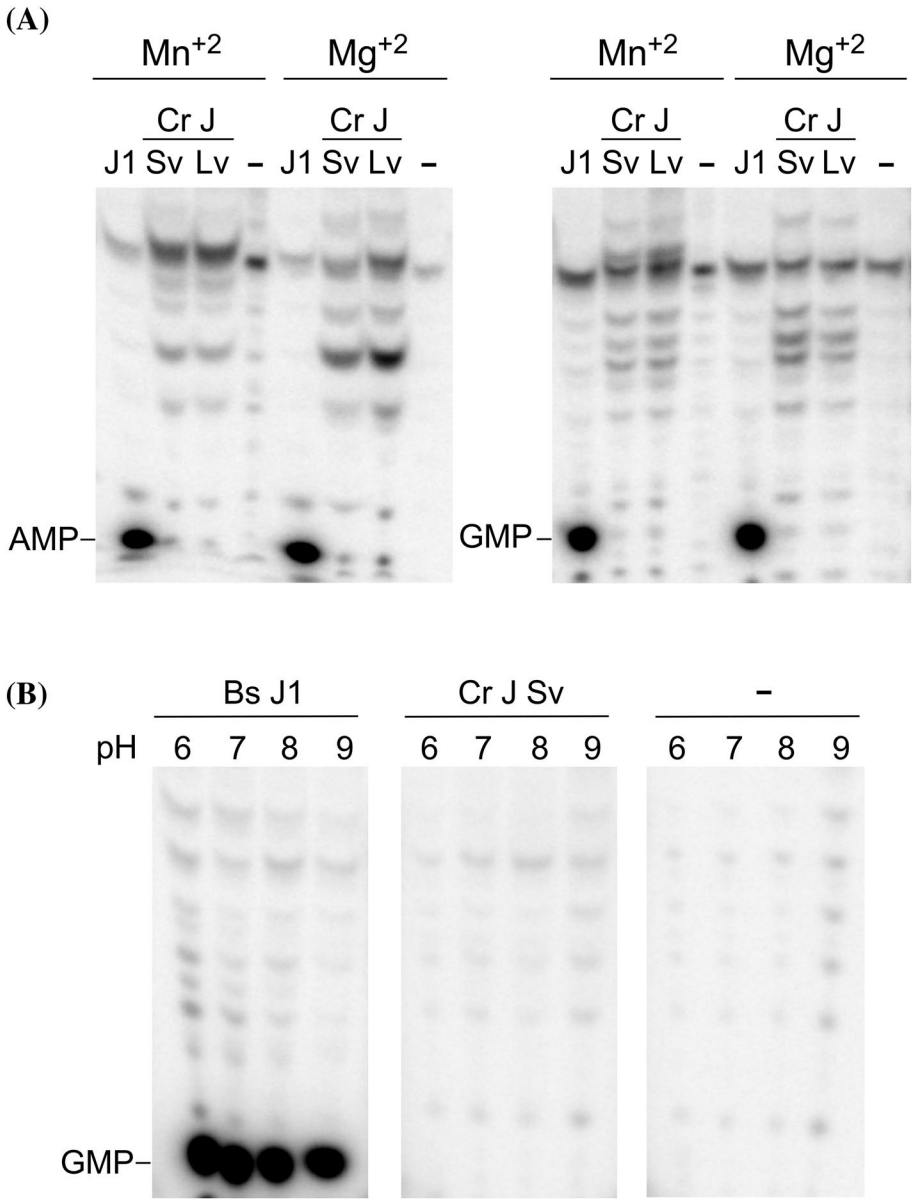

Fig. 3 Effect of divalent ions and pH on Cr RNase J activity in vitro. **a** 5′ exonuclease assay on 5′ ^{32}P end-labeled 24mer RNA transcripts (starting with A or G, respectively). J1 = Bs RNase J1, Sv and Lv = short and long version of Cr RNase J. **b** Same assay as in **a** but carried out at different pH values (pH 6–9). All reactions contained 0.75 µM of Cr RNase J

the *B. subtilis* chromosome. Addition of xylose induced the synthesis of Cr RNase J to levels similar to those observed for RNases J1/J2 in a wild type strain (Fig. 4a, ~2000–3000 molecules/cell as deduced from a Western analysis of a CR RNase J standard curve, data not shown). Induction of Cr *rnj* expression had no effect on growth of a wild type strain but could not compensate the severe growth defect caused by the lack of Bs RNase J1 (Figaro et al. 2013) (Δ*rnjA* strain, Fig. 4b top right panel). To the contrary, even low-level, leaky expression of Cr RNase J in the absence of xylose, further reduced growth in the single Δ*rnjA* mutant and also in the Δ*rnjB* mutant strain (Fig. 4b), even though the *rnjB* deletion alone has no significant growth defect. However, growth of the double Δ*rnjA/rnjB* mutant was slightly improved by the expression of Cr RNase J (Fig. 4b bottom right panel). These observations can be interpreted in terms of weak complementation and heterodimer formation (see "Discussion").

We further tested whether the inefficiency to replace Bs RNase J1/J2 might be linked to the absence of a 5′ exonuclease activity of Cr RNase J in vivo. We chose two well studied processing/maturation events that both require the 5′ exonuclease activity of RNase J1 in *B. subtilis*. In the absence of Bs RNase J1, four 16S ribosomal RNA precursors with 5′ extensions of various lengths accumulate, +38, +78, + 102, + 140 nts (Fig. 4c) (Mathy et al. 2007). Expression of Cr RNase J clearly could not compensate for the loss of Bs RNase J1, illustrated by the total absence of mature 16S rRNA (M(0), Fig. 4c, lanes 3 and 4). This strongly suggests that Cr RNase J also has no 5′ exonuclease activity in vivo, at least not in the heterologous system used here.

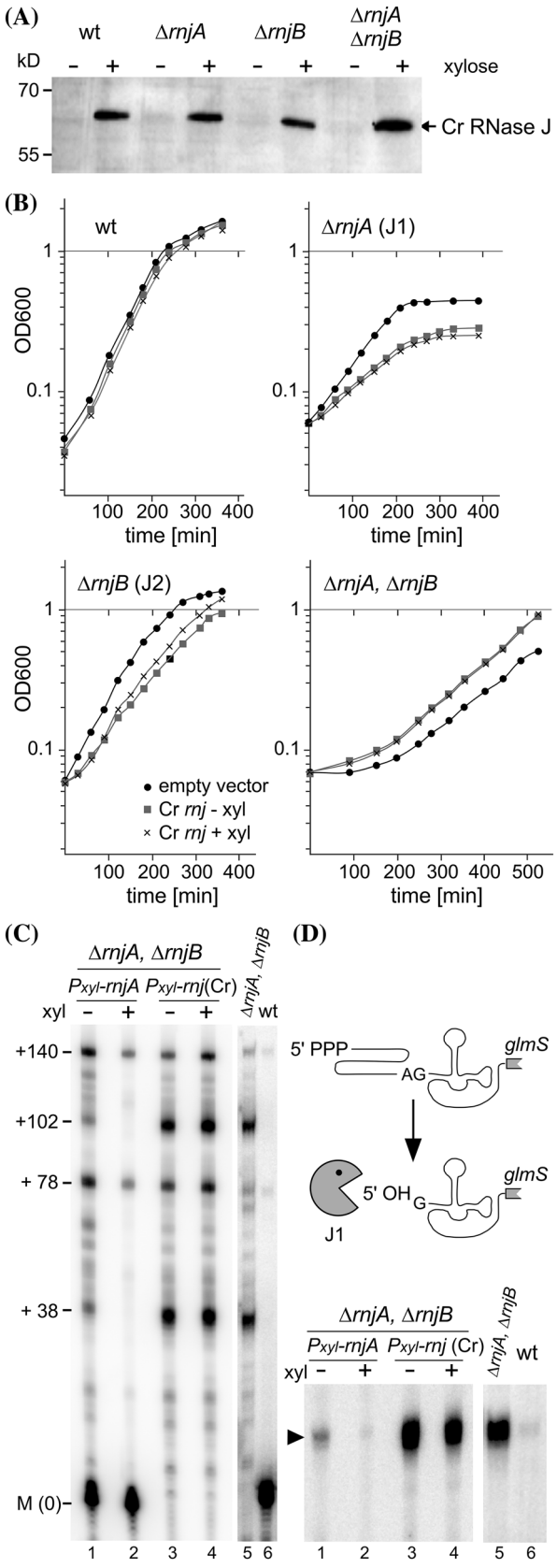

Fig. 4 Activity of Cr RNase J in vivo in a heterologous system. **a** ▶ Western blot of Cr RNase J expression from a xylose inducible promoter in *B. subtilis* wild type and RNase J1/J2 mutant strains (anti-Cr RNase J antibody). The position of Cr RNase J is indicated by an arrow. **b** Growth curves of wild type and Bs RNase J mutant strains expressing or not Cr RNase J. **c** Primer extension analysis of the effect of Cr RNase J expression on 16S rRNA maturation. The four rRNA precursors (+38, +78, +102, +140) and mature rRNA M(0) are indicated. Lanes 5 and 6 show control reactions with RNA from the *rnjA, rnjB* double mutant and a wild type strain, respectively. **d** Effect of Cr RNase J expression on the degradation of *glmS* mRNA in *B. subtilis* strains lacking RNase J1/J2. Processing of the *glmS* leader is shown schematically. Auto-catalytic cleavage occurs between an A–G liberating the *glmS* transcript with a 5′ OH. This transcript, visualized as a 190 nt primer extension product is marked by an arrowhead. Lanes 5 and 6 show control reactions with RNA from the *rnjA, rnjB* double mutant and a wild type strain, respectively

Expression of the *B. subtilis glmS* gene encoding glucosamine-6-phosphate (GlcN6P) synthase is controlled by a riboswitch/ribozyme (Collins et al. 2007). When in excess, GlcN6P binds to the 5′ UTR and induces autocatalytic cleavage at a 5′ proximal site leaving a 5′ OH group on the downstream fragment, containing the *glmS* open reading frame (cartoon in Fig. 4d). This creates an entry site for the 5′ exonuclease activity of Bs RNase J1 that rapidly degrades the downstream mRNA. As shown in Fig. 4d, the cleaved *glmS* mRNA strongly accumulates in the Δ*rnjA*, Δ*rnjB* strain (lane 5). Accumulation is weaker in the same strain expressing Bs RNase J1 from a xylose inducible gene copy even in the absence of xylose, likely due to leaky expression of Bs RNase J1 under these conditions. The processed *glmS* mRNA is almost completely eliminated after xylose induction of *rnjA* expression, similar to what is observed in a wild type strain (Fig. 4d, lane 6). By contrast, expression of Cr RNase J had no effect on the level of the *glmS* mRNA confirming that Cr RNase J has no detectable 5′ exonuclease activity when expressed in *B. subtilis*.

Role of potential co-factors for Cr RNase J activity

It seemed possible that Cr RNase J requires a co-factor to stimulate a potentially dormant 5′ exonuclease activity. We therefore carried out a yeast two-hybrid analysis to identify proteins that might interact with the nuclease. Two screens were carried out using *lexA* and *gal4* fusions. They identified a rather small number of candidate proteins. The highest possible confidence score was observed for FTT2, a member of the 14-3-3 protein family of signal-transducing adaptor proteins, considered to be an evolved member of the tetratricopeptide protein family (TPR) (Uhart and Bustos 2013). Two other high confidence candidates were mitochondrial NADH dehydrogenase (NDA3) and elongation factor EF-3 followed by a few mostly hypothetical proteins. Based on very sketchy evidence that might indicate that any of these proteins could be found in the chloroplast, we chose four

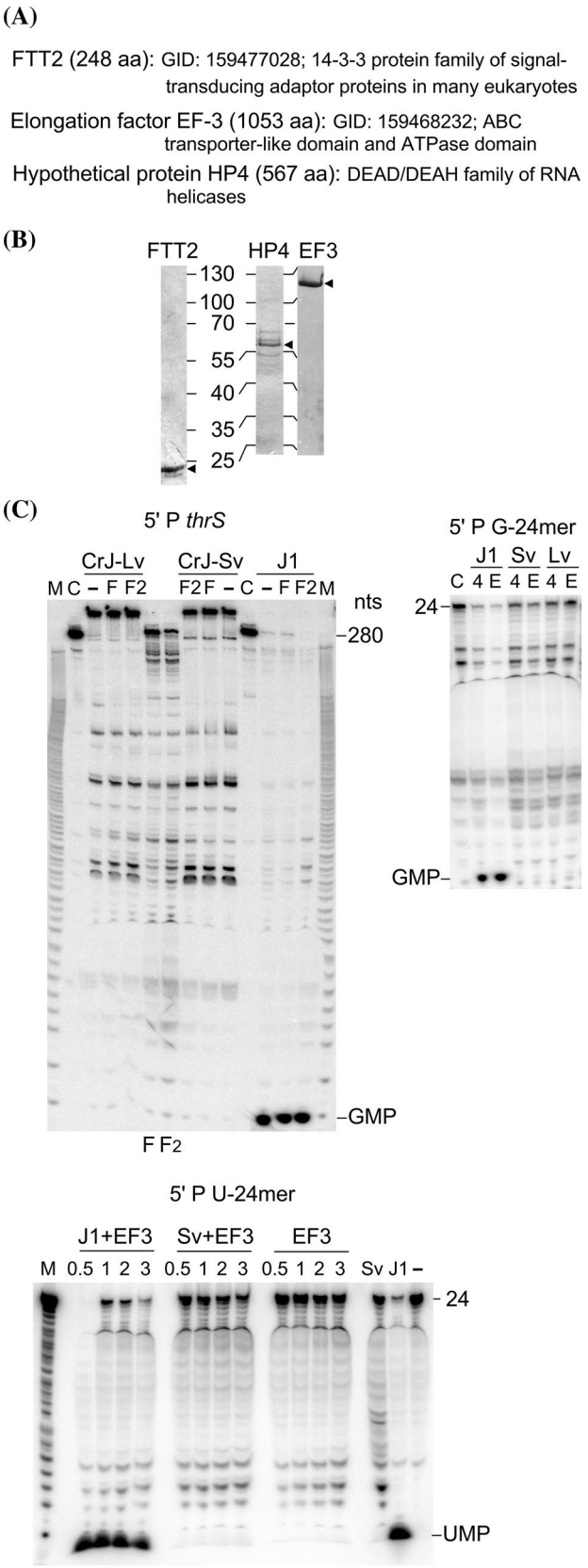

Fig. 5 Effect of Y2H candidate proteins on the Cr RNase J 5′ exonuclease activity. **a** Proteins potentially interacting with Cr RNase J identified in the yeast two hybrid screen. **b** Coomassie stained SDS-PAGE of affinity purified 6×His-tagged versions of the proteins FTT2, HP4 and EF3 described in **a** (marked by arrows). **c** Effect of the addition of the proteins FTT2, elongation factor EF-3 and hypothetical protein 4, respectively, on Cr RNase J 5′ exonuclease activity. The 5′ ^{32}P end-labeled substrates were the *thrS* leader mRNA and the 24mer RNA oligonucleotide starting with G. The full-length RNA and larger fragments are partially retained in the well due to strong binding of Cr RNase J to the RNA. The reactions contained 0.4 µM Cr RNase J or Bs RNase J1. M = marker (nt), C = control without enzyme, F and F2 = FTT2 (0.15 µM and 0.3 µM, respectively), 4 = hypothetical protein 4 (0.4 µM) E = elongation factor EF3 (0.4 µM). Numbers above lanes in bottom panel indicate the EF3 to nuclease concentration ratio

proteins for further analysis: FTT2, NDA3, EF-3 and the hypothetical protein, HP4, which is a putative RNA helicase (Fig. 5a). We were unable to sufficiently express and purify the NDA3 protein. The genes for the other three proteins were cloned, overexpressed and the proteins purified (Fig. 5b). Cr RNase J 5′ exonuclease activity was assayed in the presence of various amounts of these proteins using either the *thrS* substrate or the 5′ end-labeled monophosphorylated 24-mer RNA substrates.

As shown in Fig. 5c, none of these proteins was able to stimulate the 5′ exonuclease activity of Cr RNase J nor did they alter the endonucleolytic cleavage activity. Elongation factor EF3 is an ATPase and binding of ATP is known to induce a conformational switch in similar proteins. However, addition of a non hydrolysable ATP analog in concentrations between 0.5 and 3 mM had no effect compared to a reaction without the nucleotide (data not shown).

Discussion

A significant problem with this work was the difficulty of over-expressing *Chlamydomonas* proteins in *E. coli* for their purification. For Cr RNase J, we resolved the problem by using a codon-optimized version of the gene. In this case the overproduced proteins, both long and short versions, stayed in solution rather than forming inclusion bodies. This result clearly shows that the Rosetta and Codon+ plasmids, expressing tRNAs for the rare codons of *E. coli*, were not adequate for expressing the native *Chlamydomonas* genes. We can postulate that during translation in *E. coli* the ribosomes stall at certain codons, which allows the nascent protein to misfold so that when translation resumes, it is unable to adopt its correct structure. Instead, the misfolded protein is shunted into inclusion bodies. However, it is important to note that the proteins extracted from inclusion bodies under denaturing conditions followed by a renaturation step, had the same level of endonucleolytic activity and no significant

5′ exonucleolytic activity (data not shown) as the proteins expressed from codon optimized genes and purified under native conditions.

A significant effort was invested in trying to reveal the presumed 5′ exonucleolytic activity of Cr RNase J but without success, neither in vitro under the conditions tested nor in vivo in a heterologous system. This is surprising for at least two reasons. In all cases when it has been investigated, all RNase J proteins have a clearly detectable 5′ exonuclease activity. Notably, the closely related RNase J from *Arabidopsis* has been reported to be a dual activity enzyme (Sharwood et al. 2011). Second, current models of chloroplast mRNA 5′ end maturation, based on a number of individual studies, strongly suggest the involvement of a 5′ exonuclease activity (Barkan and Small 2014).

It is obviously complicated to prove the absence of a suspected enzymatic activity of a protein in vitro, as a doubt will remain as to whether the correct experimental conditions have been chosen. Here, we have purified different versions of Cr RNase J under native conditions. In all cases, the observed activity pattern was very similar. We are thus quite confident that Cr RNase J alone has no significant 5′ exonuclease activity. This hypothesis is clearly supported by the finding that the protein has no such activity at all when expressed in vivo at physiological concentrations in the heterologous *B. subtilis* system. Moreover, we also note that the endonucleolytic cleavage pattern was not significantly altered under the various conditions tested (see also Fig. 3).

Interestingly, Cr RNase J often cleaved endonucleolytically a transcript close to the 5′ end liberating mostly pentamers on both 5′ monophosphorylated (Fig. 2b) and 5′ triphosphorylated transcripts (data not shown). This is reminiscent of the previously proposed "sliding endonuclease" mode (Taverniti et al. 2011). In this mechanism, the native tri-phosphorylated RNA enters the RNA entry channel of the RNase J dimer and is threaded towards the active site in the same way as 5′ monophosphorylated RNA. Because the 5′ PPP moiety cannot interact productively with the phosphate-binding pocket (Li et al. 2008), the RNA could slide past the catalytic centre and be cleaved endonucleolytically between downstream nucleotides, often around 5 residues from 5′ end. With Cr RNase J, this mode of action was often observed (Figs. 2b, 3), indicating that, for some reason, the enzyme is unable to enter into exonuclease mode.

The observation that even low level expression of Cr RNase J in *B. subtilis* has a negative impact on growth of Bs RNase J1 and Bs RNase J2 single mutants, but not on the double mutant, is interesting. It suggests that the chloroplast enzyme can interact with the remaining RNase J1 or J2, which normally form a heterodimeric complex (Even et al. 2005; Mathy et al. 2010). This heterologous interaction likely leads to a less active/useful form of the protein, which impairs growth. In accordance, in the J1/J2 double mutant, where Cr RNase J is expressed alone, this effect is not observed and growth is even slightly improved. We speculate that in this case Cr RNase J does compensate at least partially for the loss of Bs RNase J1/J2 endonuclease activity. This would imply that most of the growth defect caused by the Bs RNase J1/J2 mutation is due to the missing 5′ exonuclease activity.

Our results appear to be at odds with a previous characterization of *Arabidopsis* RNase J, which was described as a dual activity 5′ exo- and endonuclease (Sharwood et al. 2011). However, in that study the 5′ exonuclease activity of the *Arabidopsis* enzyme was also very weak, especially if one considers that the RNA substrate was continuously labeled. In addition, to obtain visible amounts of mononucleotides, the RNA was incubated with the nuclease for 4 h, which is extremely long. From the available data, we estimate that the 5′ exonuclease activity of the *Arabidopsis* enzyme is not higher than the very low in vitro activity that we sometimes observed for the *Chlamydomonas* enzyme. We thus conclude that the data on both plant RNases J are not incompatible, and that chloroplast RNase J alone has no significant 5′ exonuclease activity.

In contrast to bacterial RNase J, it might actually be useful in a eukaryotic compartmentalized system to only activate a potentially deleterious activity when the enzyme has reached its final destination, in this case the chloroplast. We can thus not exclude that the 5′ exonuclease activity of Cr RNase J can be stimulated in vivo, e.g. through the interaction with a partner protein, that was not present in the heterologous *B. subtilis* system.

The yeast two hybrid (Y2H) screen yielded a number of potential interaction partners. The most pertinent candidates would, of course, be expected to not only interact with RNase J but also to localize to the chloroplast. Unfortunately, at present there is no clear evidence or a strong prediction that any of the candidates identified by the yeast two-hybrid screen actually can be found in the organelle. Therefore, we did not characterize the strength of the potential interaction of these proteins with CR RNase J, but rather tested some of them directly for their capacity to induce the 5′ exonuclease activity. The failure of the three proteins tested to do just that, by no means excludes the possibility that such factors do exist in the chloroplast. In addition, we can also not exclude the possibility that other non-proteinaceous factors or chloroplast-specific protein modifications might play a role in inducing a latent 5′ exonuclease activity in Cr RNase J.

A 5′ exoribonuclease activity still provides the most straight forward explanation for the 5′ processing of many chloroplast mRNA species. However, the task to identify the factors that might turn Cr RNase J into an exonuclease can be difficult. A reasonable approach could be to first screen the potential candidate proteins identified in the Y2H screen

for their capacity to localize to the chloroplast using GFP fusions. Alternatively, Cr RNase J might simply not function as a 5′ exonuclease and possibly resemble Bs RNase J2 in that respect which has a greatly reduced 5′ exonuclease activity compared to Bs RNase J1 (Even et al. 2005; Mathy et al. 2010). It will be worthwhile to look for other exonucleases with the same polarity. Indeed, *Chlamydomonas* encodes four paralogues of the XrnI 5′ exoribonuclease and possibly one of them might take on the role envisaged for RNase J.

Materials and methods

Bacterial strains and growth conditions

The *B. subtilis* strains used in this work are derivatives of strain SSB1002, a wild-type laboratory stock strain derived from strain 168. *B. subtilis* strains lacking the *rnjA* and/or the *rnjB* genes have total gene deletions leaving only the start and stop codons of the respective genes. All *B. subtilis* strains used in this study are listed in Table 1. *E. coli* strains JM109 and XL1-Blue were used for plasmid constructions. Protein overexpression from recombinant plasmids was carried out in *E. coli* strain BL21 carrying either the rare tRNA expressing plasmids Codon+ (Stratagen) or Rosetta (Novagen). For the complementation tests, *B. subtilis* was grown at 37 °C in SMS defined medium (Spizizen 1958). The growth curves have been measured at least three times in independent experiments. Expression of Cr RNase J was induced by adding 50 mM xylose to the culture.

When required antibiotics were added at the following concentrations: in *E. coli*: kanamycin (50 µg/ml) chloramphenicol (10 µg/ml); in *B. subtilis*: spectinomycin (100 µg/ml), neomycin (7 µg/ml), tetracycline (10 µg/ml).

Table 1 *Bacillus subtilis* strains used in this study

Strain	Genotype
SSB0506	Δ*rnjB*::Km
SSB0507	Δ*amyE*::pDR160T
SSB0507	Δ*amyE*::pDR160T Δ*rnjB*::Km
SSB0576	Δ*amyE*::pDR160T Δ*rnjA*::Tet
SSB0577	Δ*amyE*::pDR160T Δ*rnjB*::Km Δ*rnjA*::Tet
SSB0578	Δ*amyE*::pDR160T (P*xyl-rnjA*)
SSB0579	Δ*amyE*::pDR160T (P*xyl-rnjA*) Δ*rnjB*::Km
SSB0580	Δ*amyE*::pDR160T (P*xyl-rnjA*) Δ*rnjA*::Tet
SSB0581	Δ*amyE*::pDR160T (P*xyl-rnjA*) Δ*rnjB*::Km Δ*rnjA*::Tet
SSB0582	Δ*amyE*::pDR160T (P*xyl*-SVCrRNaseJ)
SSB0583	Δ*amyE*::pDR160T (P*xyl*-SVCrRNaseJ) Δ*rnjB*::Km
SSB0584	Δ*amyE*::pDR160T (P*xyl*-SVCrRNaseJ) Δ*rnjA*::Tet
SSB0585	Δ*amyE*::pDR160T (P*xyl*-SVCrRNaseJ) Δ*rnjB*::Km Δ*rnjA*::Tet

Plasmid constructs

pDR160T

Ectopic integration vector (at *amyE*) containing the xylose-inducible Psweet promoter (Rudner et al. 1998). This vector was modified by adding a transcription terminator immediately downstream of the promoter.

pHMCR8

Gene encoding Cr RNase J with optimized codons for *E. coli* and *B. subtilis* cloned in plasmid pUC57. Manufactured by Genscript.

pHMCR9

For overexpression of N-terminal His-tagged short version of Cr RNase J. PCR fragment on pHMCR8 with primers HP1808–1810 cloned in *Nde*I/*Bgl*II sites in plasmid pKYB1T.

pHMCR10

For overexpression of C-terminal His-tagged short version of Cr RNase J. PCR fragment on pHMCR8 with primers HP1782–1873 cloned in *Nde*I/*Bgl*II sites in plasmid pKYB1T.

pHMCR11

For overexpression of N-terminal His-tagged long version of Cr RNase J. PCR fragment on pHMCR8 with primers HP1808–1781 cloned in *Nde*I/*Bgl*II sites in plasmid pKYB1T.

pHMCR12

Gene encoding codon optimized ORF for protein FTT2 (GID: 5722099) with N-terminal His-tag cloned in pET-28a(+). Manufactured by Genscript.

pHMCR13

PCR fragment on the elongation factor EF3 (GID: 159468232) cDNA (Kasuza DNA Research Institute) with primers HP1849–1851 cloned in *Nde*I/*Bgl*II sites in plasmid pKYB1T.

pHMCR14

PCR fragment on the HP4 (GID: 159463464) cDNA with primers HP1855–1868 cloned in *Xba*I/*Bgl*II sites in plasmid pKYB1T.

pHMCR18

For xylose inducible expression of the short version of Cr RNase J in *B. subtilis*. PCR fragment on pHMCR8 with primers HP1779–1810 cloned in *Pac*I/*Bam*HI sites in plasmid pDR160T (SSB0578, SSB0579, SSB0580, SSB581).

pHMJ6

For overexpression N-terminal His-tagged *B. subtilis* RNase J1. PCR fragment of *rnjA* ORF (primers HP876–877) cloned in *Nco*I–*Bam*HI sites of plasmid pET28a.

pHMJ51

For xylose inducible expression of *B. subtilis* RNase J in *B. subtilis*. PCR fragment with primers HP1829–877 on *B. subtilis* SSB1002 chromosomal DNA cloned in *Pac*I/*Bam*HI sites of plasmid pDR160T plasmid.

pKYB1T

Intein fusion vector (Biolabs) used here for the overexpression of His-tagged proteins. The plasmid has been modified to contain a transcription terminator immediately downstream of the T7 promoter sequence.

Protein purification

6×His-tagged versions of Cr RNase J proteins were overexpressed in *E. coli* BL21. Typically, 100 ml cultures were grown at 37 °C in LB medium to OD_{600} of 0.5 and recombinant protein expression was induced by the addition of 0.1 mM IPTG and continuing growth for 16 h at 15 °C. Cr RNase J was affinity purified on a Ni–NTA agarose resin according to the standard protocol supplied by the supplier (QIAGEN) yielding approximately 0.8 mg of the nuclease. The eluted proteins were dialysed against buffer A (20 mM Hepes pH 8.0, 0.5 M NaCl, 10% glycerol). In order to eliminate a major contaminating protein and to separate high molecular weight and dimeric forms of the nuclease eluted from the Ni–NTA column, further purification was carried out by gel filtration. The nuclease was incubated with 5 µg/ml DNase I and 10 µg/ml RNase A for 15 min at 4 °C and loaded on a Superdex 200 column (10/300 GL, GE Healthcare) equilibrated with buffer A (flow 0.3 ml/min).

RNA in vitro transcription

In vitro transcription with T7 RNA polymerase was performed as described by the manufacturer (Promega) using a PCR fragment as template. For *thrS* leader RNA synthesis, the PCR template was prepared using oligonucleotides HP857 and 1165 (for the 280 nt transcript) or HP857 and 27 (for the synthesis of the 351 nt transcript). It carries a T7 promoter, supplied by oligonucleotide HP857, followed by *thrS* leader sequence (280 nt) ending with a transcription terminator.

RNA labelling

5′ Triphosphorylated transcripts were either continuously labelled by addition of α-(^{32}P) UTP or 5′ end labeled by the addition of γ-(^{32}P) GTP in the in vitro transcription reaction, as described previously (Shahbabian et al. 2009). The 5′ monophosphorylated transcripts were 5′ end labelled with γ-(^{32}P) ATP using T4 polynucleotide kinase (New England Biolabs). Dephosphorylation of RNA was carried out using Antarctic phosphatase (New England Biolabs). In vitro synthesized transcripts were purified from unwanted products by elution from a denaturing 8% polyacrylamide gel.

RNase cleavage assays

Cleavage reactions were carried out using either the 280 nt *B. subtilis thrS* leader RNA or 24 nt RNA oligomers (Dharmacon) which differed only in their 5′ terminal nucleotide (5′ A/C/G/U GAGAUUAAGAAAGACACA CGUAU 3′). Reactions were set up in a volume of 10 µl containing 20 mM Hepes-KOH pH 8.0, 8 mM $MgCl_2$, 100 mM NaCl, 0.24 U/ml RNasin (Promega) and 35 µM of 5′ triphosphorylated or 5′ monophosphorylated labelled RNA substrate. Alternative metal ions were tested in the same buffer, by replacing the $MgCl_2$ with either 1 mM $ZnCl_2$ or 8 mM $MnCl_2$. For measuring the influence of pH on enzyme activity Hepes-KOH buffer was replaced with Tris–HCl buffer (pH 7–9) or Citrate buffer (pH 6). RNase J concentrations were in the submicromolar range and the precise amount is indicated in the Figure legends. Unless indicated, proteins used in the assays were fresh preparations purified on Ni–NTA agarose resin (containing a mixture of multimeric and dimeric forms). The reactions were incubated for 20 min at 30 °C, and then stopped by the addition of 5 µl of 3× gel loading buffer (87.5% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue and 5 mM EDTA). Control reactions were performed by

incubating the substrate with the reaction buffer alone. In Fig. 2a, samples were extracted with phenol/$ChCl_3$ and reprecipitated prior to gel analysis to avoid strong retention of RNA by the chloroplast RNase J. The samples were analysed on 20% and/or 5% denaturing polyacrylamide gels. Radioactive signals were visualized using a Storage Phosphor Screen Bas-IP (GE Healthcare) and a Typhoon™ FLA 9500 biomolecular imager (GE Healthcare). Images were treated with Image J software.

RNA isolation

Total RNA was isolated by an adaptation of a previously published protocol (Mayford and Weisblum 1989). 25 ml *B. subtilis* cell culture was grown to $OD_{600} = 1$. Pelleted cells were washed with 1 ml of TSE buffer (100 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA) and resuspended in 500 µl of ice cold STET buffer (50 mM Tris–HCl, pH 8, 8% sucrose, 0.5% Triton X-100, 10 mM EDTA) with freshly added 3 mg/ml lysozyme, and held for 5 min on ice. Cells were mixed with an equal volume of phenol/water, vortexed for 3 min and heated at 100 °C for 1 min. The phases were separated by centrifugation, and the aqueous phase was extracted once with phenol/water (phenol pH 8), and once more with phenol/chloroform/isoamyl alcohol (25:24:1). The RNA was precipitated with ethanol-LiCl and resuspended in 25 µl water.

Primer extension reaction

Primer extension was adapted from a previously described protocol (Sambrook et al. 1989). Annealing mix containing 15 µg of total RNA, 10 U/ml RNasin (Promega) and 0.5 pmole of 5′ labelled primer in 1× hybridization buffer (300 mM NaCl, 10 mM Tris–HCl pH 7.5, 2 mM EDTA) was incubated. Primer extension reaction was started by adding 40 µl of 1.25 x RT-buffer (1.25 mM of each dNTP, 12.5 mM DTT, 12.5 mM Tris–HCl pH 8.0, 7.5 mM $MgCl_2$), 10 U RNasin (Promega) and 10 U AMV Reverse Transcriptase (Promega) to the annealing mix (10 µl), and incubated 30 min at 50 °C. The reaction was stopped by addition of 1 µl 0.5 M EDTA and 6 µl of 1 M NaOH and incubated for 10 min at 55 °C. Samples were neutralised with 6 µl of 1M HCl and precipitated with 1/10 volume LiCl 10 M, 2.5 volumes ethanol. Reaction products were separated on a 5% denaturing polyacrylamide gel. The oligonucleotides used as primers (HP1836 for 16S rRNA and HP1838 for *glmS*) were radiolabelled with γ-(^{32}P) ATP using T4 Polynucleotide Kinase (New England Biolabs). As a control for the amount of input RNA, primer extension on 5S RNA was performed using HP1837 primer.

Isolation of chloroplasts

Chlamydomonas reinhardtii cells were grown in Tris Acetate Phosphate (TAP) medium, pH 7.2 (Harris 1989) under continuous low light (10 µE m^{-2} s^{-1}). Experiments were performed using exponentially growing cells (2×10^6 cells ml^{-1}). Isolation of chloroplasts was performed as described (Zerges and Rochaix 1998). Protein isolation and immunoblotting were performed as described in Kuras and Wollman (1994). Cell extracts were loaded on an equal-chlorophyll basis.

Western blot

Western blot analysis was carried out as described previously (Jamalli et al. 2014), using an antibody directed against a peptide in the central region of Cr RNase J. This antibody does not cross-react with *B. subtilis* RNase J1/J2.

Yeast two-hybrid analysis

The yeast two-hybrid analysis was carried out by Hybrigenics (http://www.hybrigenics-services.com) using the full-length Cr RNase J as a bait and proprietary *C. reinhardtii* library.

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Author contributions AL did the cloning, protein purification and in vitro and in vivo analysis of RNase J; AJ carried out cloning of certain RNase J variants and their in vitro tests; RK and LS did the chloroplast isolation and RNase J localization experiment; EG purified and tested potential RNase J partner proteins; FAW, SL and HP were involved in experimental design, data analysis and supervision of the project. HP wrote the manuscript with support from SL.

References

Barkan A (2011) Expression of plastid genes: organelle-specific elaborations on a prokaryotic scaffold. Plant Physiol 155:1520–1532

Barkan A, Small I (2014) Pentatricopeptide repeat proteins in plants. Annu Rev Plant Biol 65:415–442

Callebaut I, Moshous D, Mornon JP, de Villartay JP (2002) Metallo-beta-lactamase fold within nucleic acids processing enzymes: the beta-CASP family. Nucleic Acids Res 30:3592–3601

Clouet-d'Orval B, Rinaldi D, Quentin Y, Carpousis AJ (2010) Euryarchaeal beta-CASP proteins with homology to bacterial RNase J Have 5′- to 3′-exoribonuclease activity. J Biol Chem 285:17574–17583

Collins JA, Irnov I, Baker S, Winkler WC (2007) Mechanism of mRNA destabilization by the *glmS* ribozyme. Genes Dev 21:3356–3368

Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 8:978–984

Even S, Pellegrini O, Zig L, Labas V, Vinh J, Brechemmier-Baey D, Putzer H (2005) Ribonucleases J1 and J2: two novel endoribonucleases in *B. subtilis* with functional homology to *E. coli* RNase E. Nucleic Acids Res 33:2141–2152

Figaro S, Durand S, Gilet L, Cayet N, Sachse M, Condon C (2013) Knockouts of the genes encoding ribonucleases RNase Y and J1 are viable in *B. subtilis*, with major defects in cell morphology, sporulation and competence. J Bacteriol 195:2340–2348

Harris E (1989) The Chlamydomonas sourcebook: a comprehensive guide to biology and laboratory use. Academic Press, San Diego

Hausmann S, Guimaraes VA, Garcin D, Baumann N, Linder P, Redder P (2017) Both exo- and endo-nucleolytic activities of RNase J1 from *Staphylococcus aureus* are manganese dependent and active on triphosphorylated 5′-ends. RNA Biol 14:1431–1443

Jamalli A, Hebert A, Zig L, Putzer H (2014) Control of expression of the RNases J1 and J2 in *Bacillus subtilis*. J Bacteriol 196:318–324

Kuras R, Wollman FA (1994) The assembly of cytochrome b6/f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*. EMBO J 13:1019–1027

Laalami S, Zig L, Putzer H (2014) Initiation of mRNA decay in bacteria. Cell Mol Life Sci 71:1799–1828

Li dela, Sierra-Gallay I, Zig L, Jamalli A, Putzer H (2008) Structural insights into the dual activity of RNase. J Nat Struct Mol Biol 15:206–212

Loiselay C, Gumpel NJ, Girard-Bascou J, Watson AT, Purton S, Wollman FA, Choquet Y (2008) Molecular identification and function of cis- and trans-acting determinants for petA transcript stability in *Chlamydomonas reinhardtii* chloroplasts. Mol Cell Biol 28:5529–5542

Luro S, Germain A, Sharwood RE, Stern DB (2013) RNase J participates in a pentatricopeptide repeat protein-mediated 5′ end maturation of chloroplast mRNAs. Nucleic Acids Res 41:9141–9151

Mathy N, Benard L, Pellegrini O, Daou R, Wen T, Condon C (2007) 5′-to-3′ exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5′ stability of mRNA. Cell 129:681–692

Mathy N, Hebert A, Mervelet P, Benard L, Dorleans A, Li de la Sierra-Gallay I, Noirot P, Putzer H, Condon C (2010) *Bacillus subtilis* ribonucleases J1 and J2 form a complex with altered enzyme behaviour. Mol Microbiol 75:489–498

Mayford M, Weisblum B (1989) Conformational alterations in the ermC transcript in vivo during induction. Embo J 8:4307–4314

Nickelsen J, Kuck U (2000) The unicellular green alga *Chlamydomonas reinhardtii* as an experimental system to study chloroplast RNA metabolism. Naturwissenschaften 87:97–107

Rudner DZ, Breger KS, Rio DC (1998) Molecular genetic analysis of the heterodimeric splicing factor U2AF: the RS domain on either the large or small *Drosophila* subunit is dispensable in vivo. Genes Dev 12:1010–1021

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Shahbabian K, Jamalli A, Zig L, Putzer H (2009) RNase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*. EMBO J 28:3523–3533

Sharwood RE, Halpert M, Luro S, Schuster G, Stern DB (2011) Chloroplast RNase J compensates for inefficient transcription termination by removal of antisense RNA. RNA 17:2165–2176

Spizizen J (1958) Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc Natl Acad Sci USA 44:407–408

Stern DB, Goldschmidt-Clermont M, Hanson MR (2010) Chloroplast RNA metabolism. Annu Rev Plant Biol 61:125–155

Tardif M, Atteia A, Specht M, Cogne G, Rolland N, Brugiere S, Hippler M, Ferro M, Bruley C, Peltier G, Vallon O, Cournac L (2012) PredAlgo: a new subcellular localization prediction tool dedicated to green algae. Mol Biol Evol 29:3625–3639

Taverniti V, Forti F, Ghisotti D, Putzer H (2011) Mycobacterium smegmatis RNase J is a 5′-3′ exo-/endoribonuclease and both RNase J and RNase E are involved in ribosomal RNA maturation. Mol Microbiol 82:1260–1276

Uhart M, Bustos DM (2013) Human 14-3-3 paralogs differences uncovered by cross-talk of phosphorylation and lysine acetylation. PLoS ONE 8:e55703

Zerges W, Rochaix JD (1998) Low density membranes are associated with RNA-binding proteins and thylakoids in the chloroplast of *Chlamydomonas reinhardtii*. J Cell Biol 140:101–110

Zhao Y, Lu M, Zhang H, Hu J, Zhou C, Xu Q, Ul Hussain Shah AM, Xu H, Wang L, Hua Y (2015) Structural insights into catalysis and dimerization enhanced exonuclease activity of RNase J. Nucleic Acids Res 43:5550–5559