FEBS Letters 582 (2008) 2577–2582

Ribonuclease activity is a common property of *Arabidopsis* CCCH-containing zinc-finger proteins

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Received 19 May 2008; revised 9 June 2008; accepted 16 June 2008

Available online 26 June 2008

Edited by Michael R. Sussman

Abstract **The CCCH class of zinc fingers occurs in a large number of *Arabidopsis* proteins. Previous studies revealed that one such protein is a nuclease, the activity of which is attributable to one of the CCCH motifs. To examine whether nuclease activity is a more general characteristic of CCCH zinc finger containing proteins, five other such *Arabidopsis* proteins were assayed for a similar activity. The results indicate that all of these proteins possess nuclease activity. Thus, nuclease activity may be a common characteristic of *Arabidopsis* CCCH-containing proteins.**

Keywords: Zinc-finger protein; RNA binding; Ribonuclease; Polyadenylation

1. Introduction

Among the most numerous of gene families in eukaryotic genomes are those that encode proteins that possess the so-called zinc-finger motifs [1,2]. One class of zinc-finger proteins is the so-called CCCH zinc-finger proteins, the CCCH motif being typically C-X8-C-X5-C-X3-H. Representatives of this family of proteins includes the factor that recognizes mRNA-destabilizing elements (tristetraprolin; [3]) and the 30-kD subunit of the mammalian Cleavage and Polyadenylation Specificity Factor (CPSF30) and its yeast relative Ythl [4]. The family of CCCH-containing proteins in plants is large [5], but few members of this family have been characterized. HUA1 is a nuclear protein that plays a role in floral morphogenesis [6,7]; this protein binds RNA and single-stranded DNA but not double-stranded DNA and acts in concert with a KH domain protein (HEN1) to regulate the processing of AGAMOUS-encoding RNAs. A less well-characterized CCCH protein, PEI1, is required for heart-stage embryo formation [8]. FES1 acts in the FRI1 pathway of flowering regulation to regulate the expression of FLC [9]. Two related proteins, AtSZFl and AtSZF2, are induced upon salt stress, and a double mutant lacking the two proteins is more sensitive than the wild-type parent to salt stress [10]. AtCPSF30 is the *Arabidopsis* ortholog of the eukaryotic polyadenylation factor subunit CPSF30, and is a calmodulin-regulated RNA-binding protein that is part of the plant polyadenylation complex [11]. Others of the 68 CCCH-containing *Arabidopsis* proteins [5] have not been characterized beyond their annotation in genome databases.

An interesting property of the *Arabidopsis* CPSF30 protein is its endonuclease activity, one that is attributable to one of the CCCH motifs of the protein [12]. That a CCCH motif is an endonucleoytic module is interesting and raises the possibility that such an activity might be more commonly found in CCCH-containing proteins than is usually thought. In this report, we describe studies that test the hypothesis that other *Arabidopsis* CCCH-containing proteins also possess nuclease activity. Our results confirm this hypothesis and raise the interesting possibility that a large number of the family of *Arabidopsis* CCCH-containing proteins are RNA-binding nucleases.

2. Methods

2.1. Sequence alignments

The amino acid sequences of *Arabidopsis* proteins possessing CCCH-type zinc-finger motifs [5] were aligned and displayed using the Clustal algorithm included in CLC Free Workbench v. 4.0.3 www.clcbio.com.

2.2. Recombinant DNA manipulations

The protein-coding regions for Ythl, Atlg04990, Atlg21570, At2g05160, At3g51950, and At5g56930 were obtained by PCR or RT-PCR[2] as described [13] using the primers and templates listed in Supplemental Table 1. PCR products were sub-cloned into pGEM-T Easy (Promega) and sequenced as described [13]. The inserts were excised from respective clones as BglII fragments and ligated into pMAL-c2 (New England Biolabs), or, in the case of Ythl, into pGEX-2 T (Pharmacia).

The zinc-finger mutants of AtSmicl were generated by using the Quick-Change site-directed mutagenesis kit (Stratagene) with pMALC2-Atlg21570 plasmid as template as per manufacturer's instructions. The results of mutagenesis were confirmed by DNA sequencing. The oligonucleotides used for the mutagenesis are listed in Supplementary Table 1.

2.3. Protein purification and assay

Recombinant proteins were affinity purified and quantified as previously described [11–13]. The GST, MBP-AtCPSF30, and maltose binding protein (MBP) preparations used here have been described in detail previously [11–13]. RNA binding was assayed using an elec-

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Abbreviations: RT-PCR, reverse transcription/polymerase chain reaction; PCR, polymerase chain reaction; MBP, maltose binding protein; SDS, sodium dodecyl sulfate

0014-5793/$34.00
doi:10.1016/j.febslet.2008.06.029

trophoretic mobility shift assay [11,12]. The labeled RNA was derived from the pea *rbcS-E9* polyadenylation signal [14]. RNA binding reactions contained 1.2-2 pmol of labeled RNA and varying amounts of protein in a volume of 10 μl. Autoradiographs were analyzed using ImageJ [15].

Nuclease activity was assayed as described previously [12] using RNA and protein concentrations indicated in the figure legends. Autoradiographs were imaged and quantitated using ImageJ.

It should be pointed out that nuclease assays were carried out for 40–45 min, while RNA binding assays were carried out for 15 min. As has been shown [12], at these shorter times, sufficient RNA remains to permit the detection of RNA–protein complexes, even with proteins possessive of nuclease activity.

3. Results

The impetus for these studies was the finding that the *Arabidopsis* ortholog of CPSF30, a CCCH-containing protein, is an endonuclease, and that this activity is associated with one of the CCCH motifs of the protein [12]. In light of reports indicating that a mouse CCCH-containing protein, Smic l, is a nuclease and might be a functional analog of CPSF30 [16], the enzymatic properties of a possible *Arabidopsis* ortholog of Smicl (Atl g21570) were examined. This protein is the closest apparent relative of Smicl amongst the 68 *Arabidopsis*

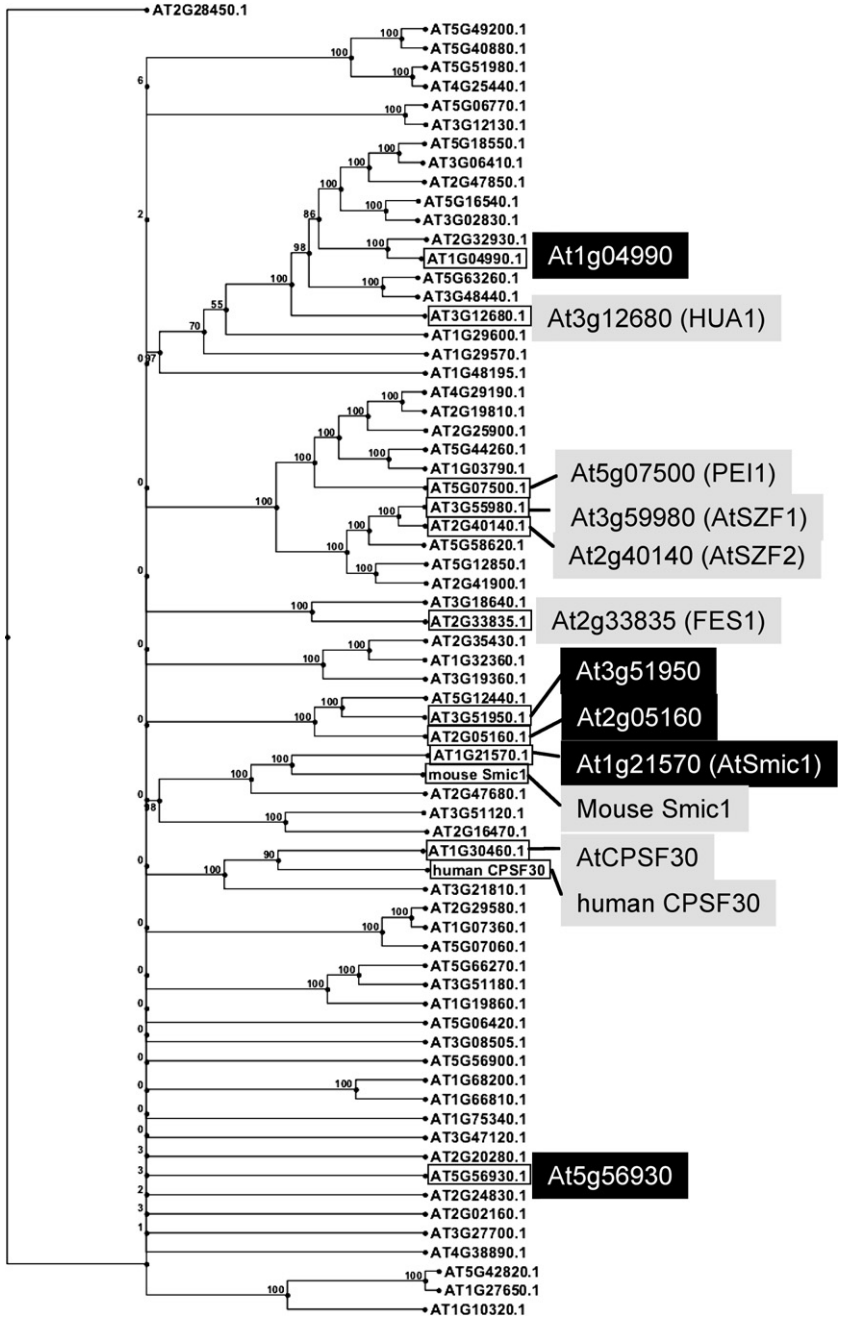

Fig. 1. Summary of amino acid alignments of the CCCH motif-containing proteins encoded by the *Arabidopsis* genome. The proteins studied in this report (At5g56930, AtSmicl, Atlg04990, At3g51950, and At2g05160) are highlighted (black boxes and white lettering). Other CCCH-containing proteins mentioned in the text (the mouse Smicl, human CPSF30, AtCPSF30, AtSZFl, AtSZF2, PEI1, and HUA1) are highlighted with gray boxes. The sequences for these proteins are provided in Supplementary File 1.

CCCH-containing proteins (Fig. 1). AtSmic l was prepared as a MBP fusion and purified using high-salt washes that have been shown to remove contaminating *E. coli*-derived nucleases [12]. The affinity-purified protein (Fig. 2A) was then assayed for RNA binding and nuclease activity. The negative control in these studies was purified MBP, while the positive control was AtCPSF30 (that is 250 amino acids in size [11].

As shown in Fig. 2B, a low-mobility RNA-containing complex formed when AtSmicl was incubated with labeled RNA (compare the lane labeled "MBP" with the lanes labeled "AtSmicl"). This result indicates that AtSmicl is able to form an RNA–protein complex. When incubated for longer times, AtSmicl degraded the RNA, while MBP had no similar effects (Fig. 2C). Therefore, like AtCPSF30 [12] and the mouse Smicl [16], AtSmicl is a nuclease.

To better understand these properties of AtSmicl, each of the five CCCH motifs of AtSmicl was altered so as to remove two of the zinc-coordinating side chains of the respective motif (Fig. 3A). After purification, four of the five proteins yielded preparations similar in quality to the wild-type AtSmicl preparations (Fig. 3B, compare lane 1 with lanes 2–5). The ZF5 mutant yielded a somewhat truncated protein (Fig. 3B, lane 5); the mobility of this protein suggested that the protein had been proteolytically cleaved in *E. coli* in or near the fifth zinc finger (not shown).

The ZF3 and ZF4 mutants possessed RNA binding activity comparable to that of the wild-type AtSmicl (Fig. 3C). In contrast, the ZF1, ZF2, and ZF5 mutants were impaired in RNA binding. The ZF3 and ZF4 mutants possessed nuclease activity comparable to the wild-type AtSmicl, and the ZF5 mutant possessed significant nuclease activity as well (Fig. 3D). In contrast, the ZF1 and ZF2 mutants had a much-reduced nuclease activity.

These results raise the possibility that other CCCH-containing proteins might also be nucleases. To test this, four other *Arabidopsis* CCCH-containing proteins (depicted in Fig. 4A) were assayed for RNA-binding and nuclease activities. These proteins were selected to be somewhat representative of the range of *Arabidopsis* CCCH-containing proteins (Fig. 1). In the process of cloning one cDNA (At5g56930), two splice variants were readily and consistently seen (not shown). This variant involved alternative splicing using splice sites within the second and sixth exons of the "normally-spliced" mRNA (Fig. 4B), and the resulting mRNA is predicted to encode a protein that has a deletion of 177 amino acids but retains the three CCCH motifs. The results of amplification of the other four genes were as predicted from the *Arabidopsis* genome information (not shown).

These five proteins were prepared as MBP fusion proteins and the preparations assessed by SDS–PAGE (Fig. 4C). The results showed a range of yields, with the large At5g56930 isoform being present in the lowest quantities. While considerable quantities of partial proteins were seen in some of these preparations, the quantities of full-sized proteins in these were enough to permit further study. One protein, At2g05160, was apparently subject to proteolysis; comparison with a deletion mutant that retains the zinc finger (not shown) indicated that the protein analyzed in Fig. 4C contains the CCCH zinc finger shown in Fig. 4A. Because the major interest of this study was activities associated with the CCCH zinc fingers, this preparation was included in the studies that follow. For this study, a GST-Yth l fusion protein was also produced (Fig. 4C, lane 6); Ythl is the yeast ortholog of CPSF30, and was used here as a non-plant CCCH-containing protein.

As shown in Fig. 4D, the proteins encoded by Atlg04990 (Fig. 4D, lane 1) and At3g51950 (Fig. 4D, lane 4), and Ythl (Fig. 4D, lane l0) each possessed RNA-binding activity. In addition, the larger isoform encoded by At5g56930 also was able to bind RNA (Fig. 4D, lane 2). These three proteins all bound RNA with similar apparent affinities (Fig. 4E). While saturation was not reached with one of these (At3g51950), it is apparent from this study that the apparent affinities for these three proteins are in the 0.1–1 μM range.

The smaller At5g56930-derived isoform was a poor RNA-binding protein (Fig. 4D, lane 3). RNA binding activity was not seen with the At2g05160-encoded protein (Fig. 4D, lane 5), probably due to the combined circumstances of the truncation of this protein (as depicted in Fig. 4A) and the excessive nuclease activity of the protein (see the following).

As shown in Fig. 4F, the proteins encoded by Atlg04990 and At2g01560 possessed considerable nuclease activity, more than comparable to that of AtSmic l (Atl g21570 in Fig. 4F) under the assay conditions. The other three proteins also possessed nuclease activity, albeit some 30–50% lower under these conditions than that seen with AtSmic l. Affinity-purified GST-Ythl, the yeast ortholog of AtCPSF30, was devoid of nuclease activ-

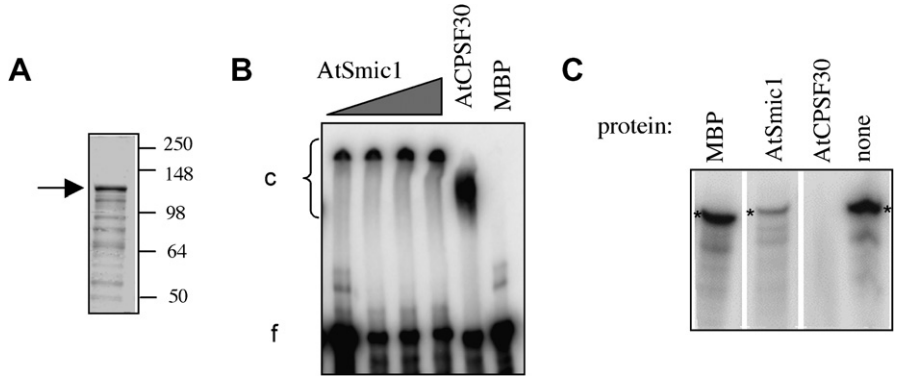

Fig. 2. Activities of AtSmicl. (A) Coomassie Brilliant blue stained gel showing the affinity purified MBP-AtSmicl. The full-sized MBP-AtSmicl is denoted with an arrow. (B) RNA binding by AtSmicl. Labeled RNA (200 nM) was assayed with AtSmicl protein (0.7, 1.2, 1.6, and 2.4 μM, from left to right), AtCPSF30 (3.5 μM), or MBP (4.0 μM). RNA–protein complexes formed in each reaction are denoted by "c" and free RNA by "f". (C) Nuclease activity of AtSmicl. Labeled RNA (200 nM) was incubated with AtSmicl (200 nM), AtCPSF30 (300 nM), or MBP (300 nM) at 30 °C for 40 min. The RNAs were recovered and analyzed on sequencing gels. The substrate RNA is denoted by "*".

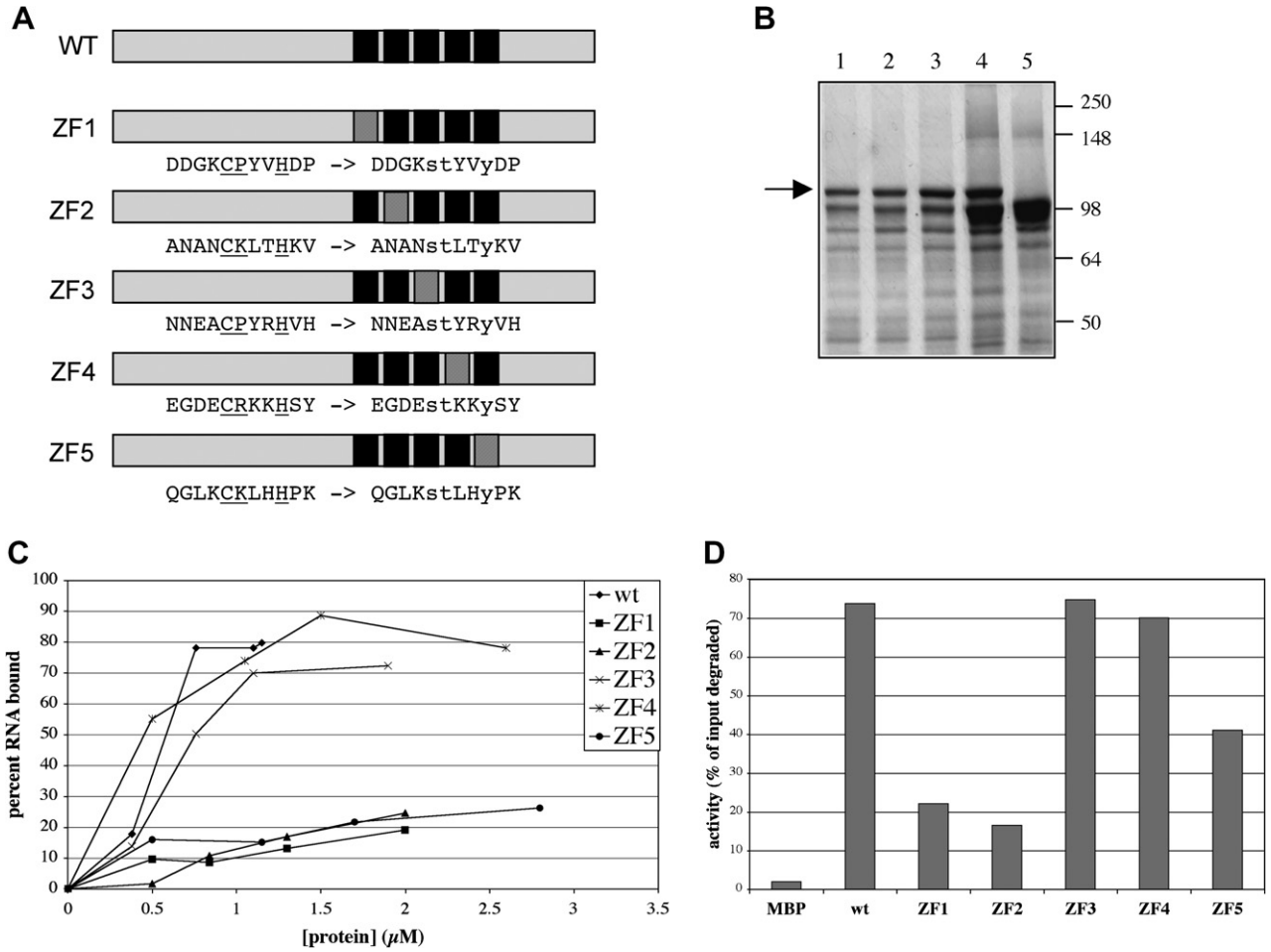

Fig. 3. Mutational analysis of AtSmicl. (A) Illustration of AtSmicl and various zinc-finger mutants. Unmodified zinc fingers (black bars), mutated zinc fingers (hatched bars), and the corresponding wild-type (left, underlined) and mutant (right, lower case) amino acid sequences are indicated beneath each mutant depiction. (B) Stained gel showing various affinitypurified protein preparations. Lanes: 1 – ZF1; 2 – ZF2; 3 – ZF3, 4 – ZF4; 5 – ZF5. The full length polypeptide is denoted by arrow. The positions of protein size standards are shown beside the gel. (C) RNA binding assays. RNA-binding activity is represented as the fraction of RNA present in complexes, and is plotted as a function of protein concentration. The RNA concentration in these reactions was 120 nM. (D) Nuclease assays. Labeled RNA (120 nM) was incubated with the indicated proteins for 45 min at 30 °C and the remaining RNA recovered and analyzed on a sequencing gel. The percentage of input RNA degraded was plotted as shown. The identity of the AtSmicl variant is indicated along the *X*-axis. The quantities of the enzyme preparations used in the assays were: 'wt' (wild-type AtSmicl) – 19 pmol; ZF1 – 21 pmol; ZF2 – 21 pmol; ZF3 – 19 pmol; ZF4 – 20 pmol; ZF5 – 21 pmol.

ity in this assay; this is not due to the possibility that this protein was purified in inactive form, since the same preparation binds RNA (Fig. 4D, lane 10).

The purified GST and MBP proteins also showed no detectable nuclease activity. These controls demonstrate the effectiveness of the purification protocol in removing contaminating *E. coli* nucleases, and indicate that the *Arabidopsis* CCCH proteins are nucleases. Moreover, the Yth l control indicates that not all CCCH-containing proteins are nucleases. Rather, this activity is a property of particular CCCH proteins, and may in fact distinguish between them.

4. Discussion

In *Arabidopsis*, the CCCH family of zinc-finger proteins is sizeable (Fig. 1; [5]). As was seen with AtCPSF30 [12], the five CCCH proteins assayed in this study possess nuclease activity. A number of observations argue that these activities are inherent properties of the respective proteins, and not artifacts. The absence of nuclease activity of the control protein preparations (MBP and GST) as well as the Ythl protein (Fig. 4) shows that non-specifically associated *E. coli* nucleases are not retained by the affinity matrices used to prepare the proteins. The observations that mutation of subsets of the CCCH motifs present in AtCPSF30 [12] and AtSmicl (Fig. 3) reduce or eliminate nuclease activity also argue against an involvement of contaminating enzymes in the activities seen here; such artifacts should not be affected by specific mutations in the CCCH motifs. The effects of these mutations moreover support the hypothesis that specific CCCH zinc-finger motifs in and of themselves are nucleolytic. Taken together, these results raise the interesting possibility that many of the *Arabidopsis* CCCH motif-containing family of proteins may be ribonucleases. Such an eventuality would necessitate considerable revision of our conception

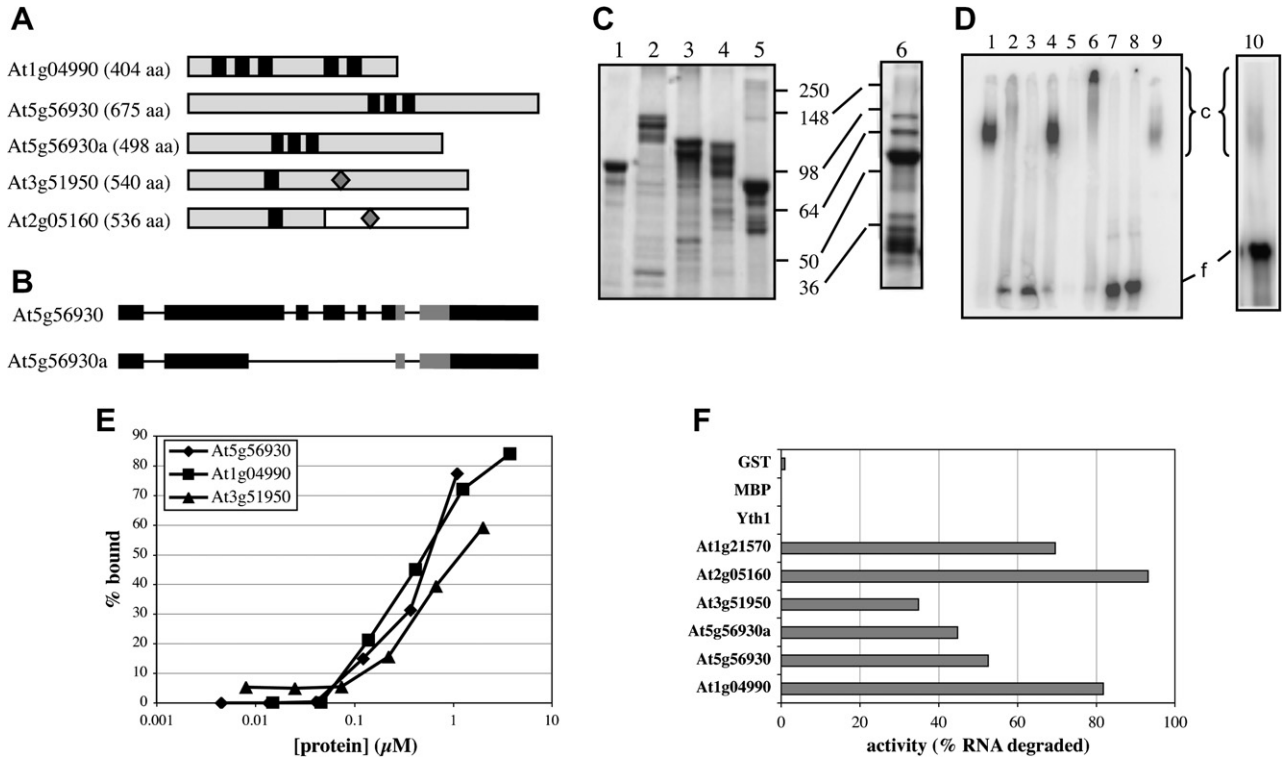

Fig. 4. Activities of *Arabidopsis* CCCH-containing proteins. (A) Illustration of the proteins of interest. The protein-coding regions (shaded rectangles), zinc-finger motifs (thick black boxes) and RRM domains (grey shaded diamonds) are shown for each protein. For At2g05160, the part of reading frame present in the purified MBP fusion protein (shaded portion) and the portion that is removed by proteolysis (white box) during growth and/or purification are also depicted. (B) Splice variants of At5g56930 transcripts. Exons (dark rectangles), introns (thin lines), and zinc finger (grey rectangles) are shown. C. Stained gel showing each purified protein preparation. Lane l – Atlg04990, lane 2 – At56930, lane 3 – At5g56930a, lane 4 – At3g51950, lane – 5 At2g05160; lane 6 – GST-Ythl. Size standards are indicated with small horizontal tics, and the corresponding sizes shown. (D) RNA-binding assays. Labeled RNA (120 nM for MBP and the CCCH proteins, 135 nM for Ythl) was incubated with the indicated proteins and RNA binding was assessed. Lane 1 – Atlg04990 (1.5 μM), lane 2 – At5g56930 (1.3 μM), lane 3 – At5g56930a (1.9 μM), lane 4 – At3g51950 (1.6 μM), lane 5 – At2g05160 (1.9 μM), lane 6 – AtSmicl (2.0 μM), lane 7 – MBP (2.2 μM), lane 8 – RNA only, lane 9 – AtCPSF30 (2.0 μM), lane 10 – GST-Ythl (7.8 μM). Purified GST did not bind RNA under these conditions (not shown; [13]). (E) RNA binding by the At5g56930, Atlg04990, and At3g51950 proteins as a function of protein concentration. Labeled RNA (120 nM) was incubated with the indicated concentrations of proteins, and RNA binding assayed. Activity is represented as the percent of RNA that is bound. (F) Nuclease assays. RNA (120 nM for MBP and the CCCH proteins, 135 nM for GST and Ythl) was incubated with purified fusion proteins for 45 min (for MBP and the *Arabidopsis* CCCH proteins) or 60 min (for GST and the Ythl fusion protein) at 30 °C and the remaining RNA recovered and analyzed on a sequencing gel. Activity is represented as the percent of substrate that is degraded. Protein quantities used in these assays: Atlg04990 – 10.5 pmol; At5g56930 – 10 pmol; At5g56930a – 12 pmol; At3g51950 – 11 pmol; At2g05160 – 7.4 pmol; AtSmicl – 12 pmol; MBP – 22 pmol; Ythl – 13 pmol; GST – 13 pmol.

of these proteins, that are usually thought of as nucleic acid (DNA or RNA) binding.

The results presented here also reveal interesting distinctions between AtCPSF30 and other CCCH-containing proteins. In the case of AtSmicl, more than one CCCH motif is needed for RNA-binding (the 1st, 2tnd and 5th) and nuclease (the 1st and 2nd) activity, and there is a coincidence of RNA-binding and nuclease-associated CCCH motifs. AtCPSF30, on the other hand, has different and distinct RNA-binding and nuclease-associated zinc-finger motifs [12]. Interestingly, with both AtSmicl and AtCPSF30, mutants impaired in RNA binding retain their nuclease activities (Fig. 3; [12]). This does not necessarily mean that the nuclease-associated zinc-finger motifs do not also bind RNA in the course of nuclease action; it is probable that RNA binding by the nucleolytic zinc fingers is too transitory to be detectable by the gel-shift assay. Instead, these studies indicate that the RNA binding that is apparent in the gel-shift assays are activities distinct from nuclease activities. Taken together, these observations are suggestive of multiple biochemical functions for these two proteins.

The proteins encoded by At5g56930 are also interesting. While both isoforms possess nuclease activity, only the larger is an RNA-binding protein. This implicates sequences apart from the zinc fingers in RNA binding, and raises interesting questions as to the possible functional significance of the alternative splicing of At5g56930-encoded mRNAs.

Possible molecular functions for CCCH-associated nucleases can only be speculated on. One possible role is that which was the impetus of the present study, namely functional analogs of CPSF30 in mRNA 3′ end formation. The mouse Smicl protein is an Smad-interacting CCCH-containing protein that can function *in vitro* as a CPSF30-like activity in 3′ end processing [16]. The similarity between Smicl and AtSmicl is suggestive that the latter may be a functional analog of AtCPSF30. That both proteins are RNA-binding nucleases supports this hypothesis.

Bacteria possess sets of ribonuclease/anti-ribonuclease pairs that have been implicated in programmed cell death, stress responses, and other processes that may require a shut-down of gene expression [17,18]. It is probable that the nucleases identified here also possess (as yet unidentified) inhibitors that serve

to limit the activity of the nucleases to specific substrates and times. This follows from the observation that uncontrolled or unregulated ribonuclease expression is highly toxic to plant cells [19,20], and the absence of detectable extracellular or vacuolar targeting information amongst the set of *Arabidopsis* CCCH-containing proteins. One example of such a pair is the AtCPSF30-AtFip(V) proteins in *Arabidopsis;* it has been shown that AtFipl(V), an *Arabidopsis* ortholog of the yeast polyadenylation factor subunit Fipl [21] inhibits the nuclease activity of AtCPSF30, presumably by binding to the nucleolytic zinc finger [12].

In summary, these experiments reveal the existence of a family of heretofore unrecognized ribonucleases in *Arabidopsis*, the size of which may be quite considerable. These results introduce a new character into any discussion of the functioning of CCCH-containing proteins in plants and other organisms.

Acknowledgements: The authors thank Carol Von Lanken for excellent technical and administrative support. This work was supported by NSF *Arabidopsis* 2010 Grant MCB-0313472.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.06.029.

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