SHORT COMMUNICATION

VaSpoU1 (SpoU gene) may be involved in organelle rRNA/tRNA modification in *Viscum album*

Joon-Woo Ahn · Suk Weon Kim · Jang Ryol Liu ·
Won-Joong Jeong

Abstract The SpoU family of proteins catalyzes the methylation of transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs). We characterized a putative tRNA/rRNA methyltransferase, VaSpoU1 of the SpoU family, from *Viscum album* (mistletoe). VaSpoU1 and other plant SpoU1s exhibit motifs of the SpoU methylase domain that are conserved with bacterial and yeast SpoU methyltransferases. *VaSpoU1* transcripts were detected in the leaves and stems of *V. album*. VaSpoU1-GFP fusion proteins localized to both chloroplasts and mitochondria in *Arabidopsis* protoplasts. Sequence analysis similarly predicted that the plant SpoU1 proteins would localize to chloroplasts and mitochondria. Interestingly, mitochondrial localization of VaSpoU1 was inhibited by the deletion of a putative N-terminal presequence in *Arabidopsis* protoplasts. Therefore, VaSpoU1 may be involved in tRNA and/or rRNA methylation in both chloroplasts and mitochondria.

Keywords Methyltransferase · SpoU family · Subcellular localization · AdoMet (S-adenosyl-$_L$ methionine) · SpoU methylase domain

J.-W. Ahn · J. R. Liu · W.-J. Jeong (✉)
Plant Systems Engineering Research Center,
Korea Research Institute of Bioscience and Biotechnology
(KRIBB), 111 Gwahangno, Yuseong-gu,
Daejeon, 305-806, Korea
e-mail: wonjoong@kribb.re.kr

S. W. Kim
Microbial Resource Center, Korea Research Institute
of Bioscience and Biotechnology (KRIBB),
111 Gwahangno, Yuseong-gu, Daejeon, 305-806, Korea

Introduction

RNA modification is a universal process for RNA maturation. Ribose methylation of ribosomal RNA (rRNA) and transfer RNA (tRNA) may function to fine-tune the three-dimensional RNA folding that is required for ligand interactions, optimal translation, and assembling of the ribosomal subunits (King et al. 2003; Lane et al. 1995; Pintard et al. 2002; Sirum-Connolly et al. 1995). Approximately 107 different RNA modifications have been reported (http://rna-mdb.cas.albany.edu/RNAmods/), and pseudouridine and 2′-*O*-methylation of nucleosides are classified as common types of RNA modification. 2′-*O*-Methylation of both rRNA and tRNA can be catalyzed by the α/β-knot superfamily of methyltransferases. The SpoU family, one subfamily of the α/β-knot superfamily, is involved in ribose methylation of rRNA and tRNA (Bateman et al. 2004; Persson et al. 1997; Sirum-Connolly and Mason 1993; Thompson et al. 1982). Members of the SpoU family of proteins share common motifs in their SpoU methylase domains (Gustafsson et al. 1996; Watanabe et al. 2005, 2006).

rRNA methylation mediated by SpoU family members has been reported in *E. coli*, in which RlmB catalyzes the methylation of guanosine 2251 in the 23S rRNA (Lovgren and Wikstrom 2001). In *Saccharomyces cerevisiae*, Pet56 (a RlmB ortholog) catalyzes the methylation of G2270 in the mitochondrial 21S rRNA (Sirum-Connolly and Mason 1993). SpoU family members are also associated with tRNA methylation. For example, *E. coli* TrmH (Gm18; classical name: SpoU; EC 2.1.1.34) catalyzes the transfer of the methyl group from AdoMet (S-adenosyl-$_L$ methionine) to the 2′-OH group of guanosine at G18 in tRNA (Gustafsson et al. 1996; Persson et al. 1997). tRNA Gm18 is also modified in other bacterial organisms, such as

Thermus thermophilus and *Aquifex aeolicus* (Hori et al. 2002, 2003). In addition, TRM3, homologous to *E. coli* TrmH, catalyzes the methylation of tRNAs at G18 in *S. cerevisiae* (Cavaille et al. 1999). G18 modification of organellar tRNA in plant chloroplasts and mitochondria has been detected; however, the gene(s) responsible has not yet been identified (Francis and Dudock 1982; Marechal-Drouard et al. 1990; Marechal et al. 1985; Pirtle et al. 1981; Rozenski et al. 1999). A recent study examined Gm18 modification of tRNA by AtTRM3 (a putative homolog of yeast TRM3) using T-DNA knock-out mutants in *Arabidopsis*. However, no significant differences in Gm content were detected between wild-type and mutant plants (Chen et al. 2010).

This is a first report for characterization of plant VaSpoU1 in *Viscum album* (mistletoe; hemi-parasitic plant) which grows on the stems of various woody plants such as poplar and pine trees. VaSpoU1 and other plant SpoU1s exhibit SpoU methylase domain motifs that are conserved in bacterial and yeast SpoU proteins. VaSpoU1 was localized to chloroplasts and mitochondria. These results suggest that VaSpoU1 may be involved in tRNA and/or rRNA processing in both of these organelles.

Materials and methods

Sample collection and RNA isolation

V. album L (var. *coloratum* [Kom.] Ohwi) samples were collected from the Deok-Yu Mountains in Korea. Total RNA was extracted from *V. album* leaves and stems using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. To remove polysaccharides, total RNA was resuspended in DEPC water containing 1% SDS. Genomic DNA was removed using RNase-free DNase (Ambion, Germany) and purification was performed according to the manufacturer's instructions.

Identification of plant SpoU family members and prediction of subcellular localization

The program Position-Specific Iterative BLAST (PSI-BLAST) from NCBI was utilized to identify plant SpoU1s using the amino acid sequence of VaSpoU1 (AAR88654) as the query sequence. To identify other plant SpoU family members, *Arabidopsis* protein precursors annotated SpoU family member were used as query sequences. The ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html/) from EMBL-EBI was used for sequence alignments of SpoU family members. To predict the subcellular localization of SpoU1 proteins, PSORT and WoLF PSORT programs (http://psort.hgc.jp/ and http://wolfpsort.org/) were utilized.

5′-RACE PCR

First-strand cDNA synthesis was performed with gene-specific primer (GSP) 1 using SuperScript^{TM} II RT (Invitrogen) according to the manufacturer's instructions. TdT tailing of *VaSpoU1* cDNA was performed with 200 μM dCTP and TdT at 37°C for 10 min. The initial PCR was performed with 400 nM of each GSP 2 and anchor 1 primer PCR products were diluted 500-fold for use as templates for the nested PCR. Nested PCR was conducted with 1 μl of diluted PCR product and 200 nM of each anchor 2 and GSP 2 primers. The following program was used for both PCRs: 35 cycles of 92°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Primers used for 5′-RACE PCR were: GSP 1, 5′-AAC GTC GAC TTT CAT ACC TCC AGC TTC ACC ATG CAT CAT T-3′; GSP 2, 5′-AAT AAA CGG ATC CAG GGC TTC-3′; anchor 1, 5′-GGA CAC GCG TCG ACT AGT ACG GGG GGG GGG-3′; anchor 2, 5′-GGA CAC GCG TCG ACT AGT AC-3′.

Quantitative PCR

Reverse transcription (RT) was performed using 2 μg total RNA and 200 units M-MLV reverse transcriptase (Promega, USA), 50 μM oligo(dT), 500 μM of each dNTP, and 20 units of ribonuclease inhibitor. For real-time RT-PCR, EvaGreen Real-Time PCR kits (SolGent, Korea) were utilized according to the manufacturer's instructions with a light cycler (Opticon2; MJ research). The following program was followed: 40 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 30 s. *VaActin* (JF461053) served as an internal control. Primers used were: *VaActin*, 5′-ACG ATG TTC CCT GGC ATT GCA-3′ and 5′-CTC ACT ACT TGA TGG CAC TT-3′; *VaSpoU1*, 5′-TGG CTG GACAT TGA ATT GT-3′ and 5′-TCT AGC AGC ATG CTC ATG A-3′.

Generation of GFP fusion constructs

VaSpoU1 cDNAs, corresponding to the N-terminal coding regions (amino acids 1–120 and 24–120), were PCR amplified with gene-specific primers. These cDNAs were cloned into a 326-GFP vector (Lee et al. 2001) using the *Bam*HI site. The primers used were: VaSpoU1-1F, 5′-ACC GGA TCC AAA GAG TAA AGA AGA ACA GGA TGA AAG CTT GCA AAG CC-3′; VaSpoU1-2F, 5′-GGC CGG ATC CAA AGA GTA AAG AAG AAC AGG ATG ATT TCT TCA TCT TTC-3′; VaSpoU1-120R, 5′-GCC GGA TCC TTG CTT CTA GCT TTC TTT CCA TTT TCA TCA-3′.

Transient expression of VaSpoU1-GFP in *Arabidopsis* protoplasts

For transformation of the fusion constructs into *Arabidopsis* protoplasts, 20 μg of plasmid DNA from each fusion construct was mixed with 250 μl of the protoplast suspensions (5×10^6 cells ml^{-1}) and 250 μl polyethylene glycol. Samples were incubated for 20 min at room temperature, washed with W5 solution, and incubated at 24°C for 24 h. Fusion protein expression was observed by confocal laser scanning microscopy (Carl Zeiss LSM 410). The wavelengths used were: excitation 488 nm and emission 505–530 nm for green fluorescent protein, excitation 488 nm and emission 650 nm for chlorophyll autofluorescence, and excitation 558 nm and emission 584 nm for red fluorescent protein.

Results

Isolation and identification of *V. album* VaSpoU1

We obtained partial sequences (ESTs) of *VaSpoU1* and *VaActin* of *V. album* from the Biological Resource Center in KRIBB (unpublished). Based on the partial sequence, full-length *VaSpoU1* cDNA was isolated using 5′-RACE PCR. *VaSpoU1* (AY428735) encodes a 368-amino acid protein with an estimated molecular weight of

Fig. 1 Comparison of VaSpoU1 with bacteria and yeast SpoU proteins. **a** Schematic diagram of SpoU structures. *Gray boxes* indicate SpoU methylase domains. **b** Amino acid sequence alignment of VaSpoU1 with *E. coli* and *S. cerevisiae* SpoU proteins. *Gray symbols* indicate conserved residues in the motifs. This figure is based on Watanabe et al. (2006). Pet56 (*S. cerevisiae*, CAA99414); RlmB (*E. coli*, AP_004680); TrmH (*E. coli*, AP_004141)
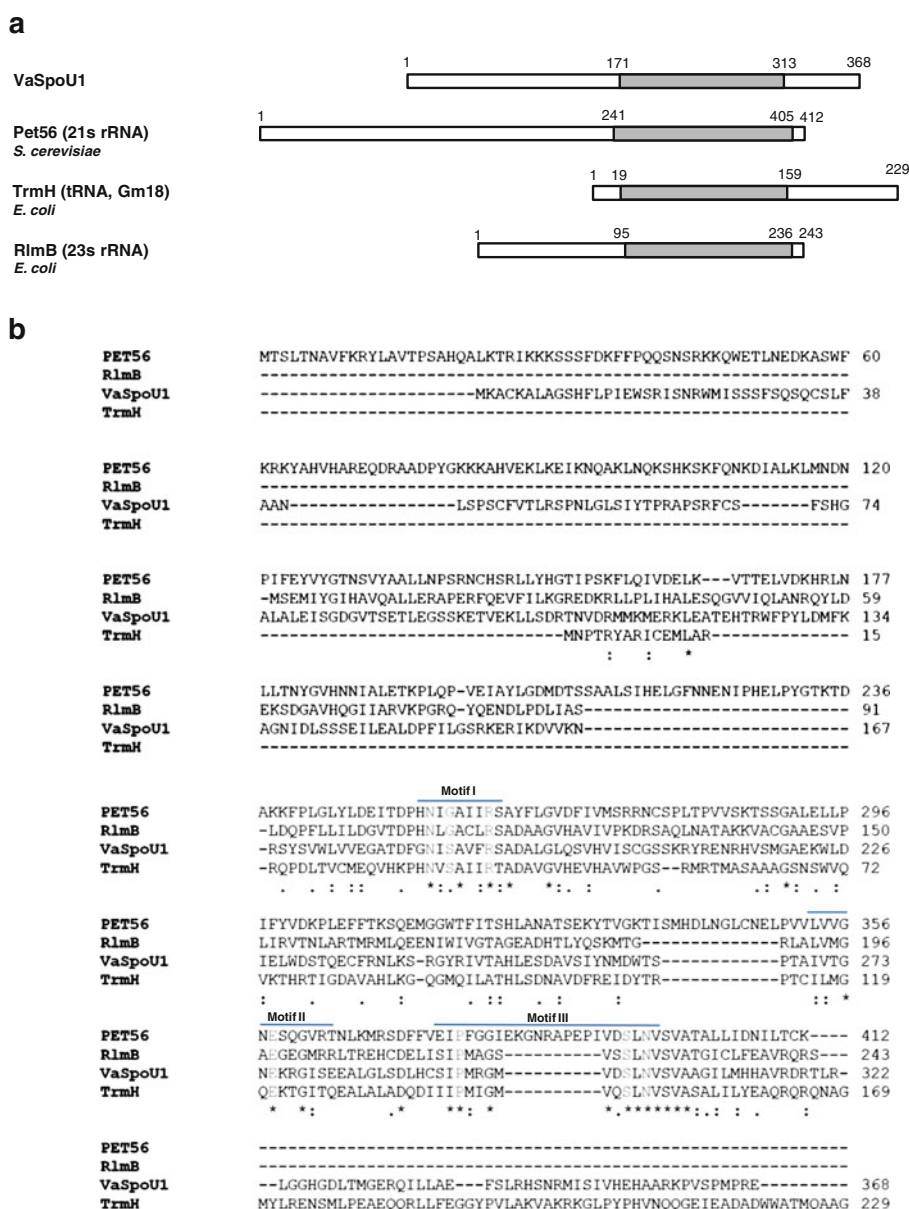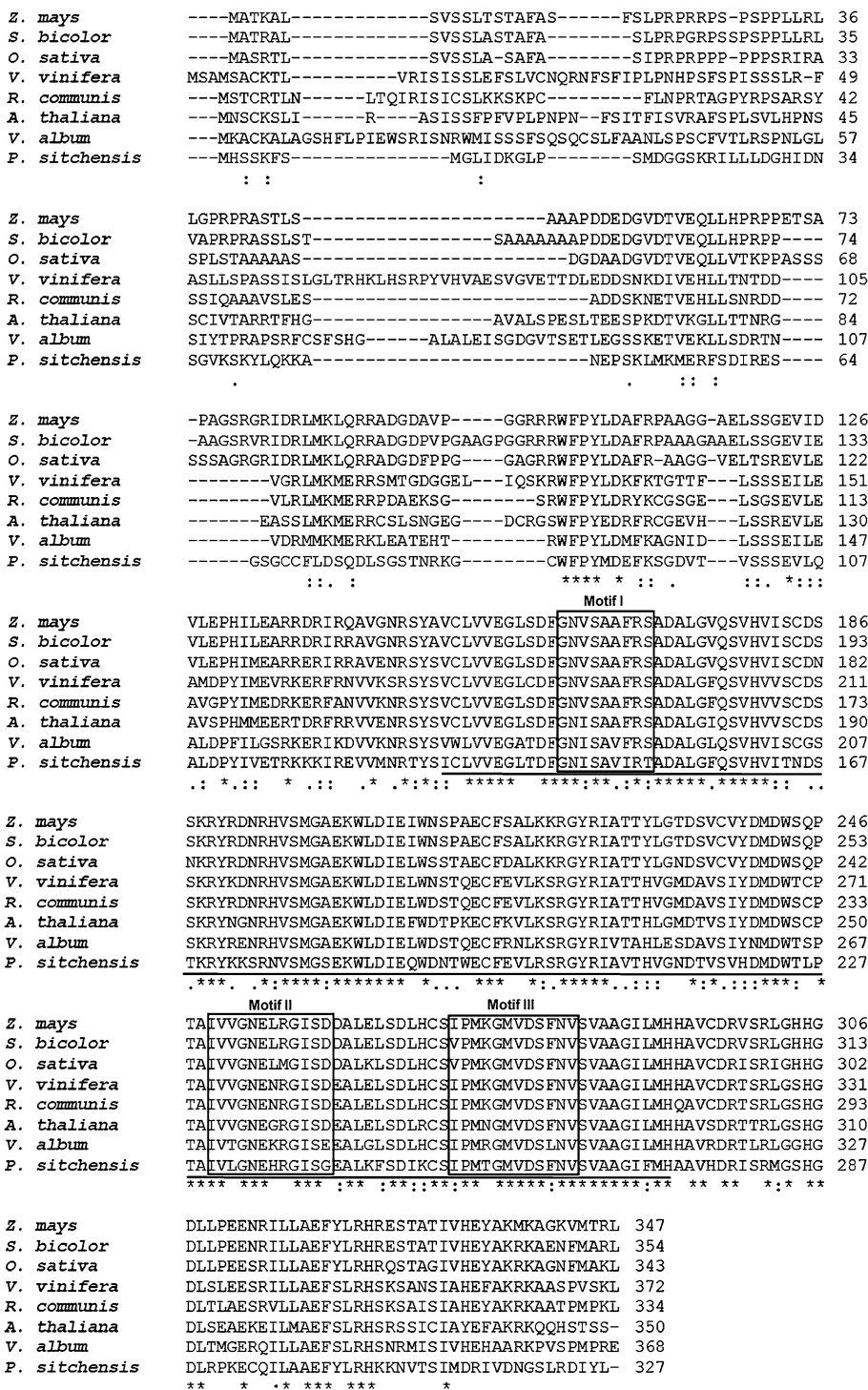
41,069.89 Da. As shown in Fig. 1a, the VaSpoU1 precursor protein is larger than the *E. coli* SpoU proteins RlmB (243 aa) and TrmH (229 aa), and smaller than *S. cerevisiae* Pet56. Motif analysis of VaSpoU1 revealed that its SpoU methylase domain shares conserved motifs with TrmH, RlmB, and Pet56 (Fig. 1b). Pet56 and RlmB have N-terminal extensions from the SpoU methylase domain, whereas TrmH has a C-terminal extension. Interestingly,

both N- and C-terminal extensions from the SpoU methylase domain are found in VaSpoU1.

Identification of plant SpoU1s and their conserved motifs

PSI-BLAST searches using the NCBI protein database were performed to identify additional plant SpoU1s. Six

Fig. 2 Sequence alignment of plant SpoU1s. *Lines* and *boxes* indicate SpoU methylase domains and its motifs, respectively
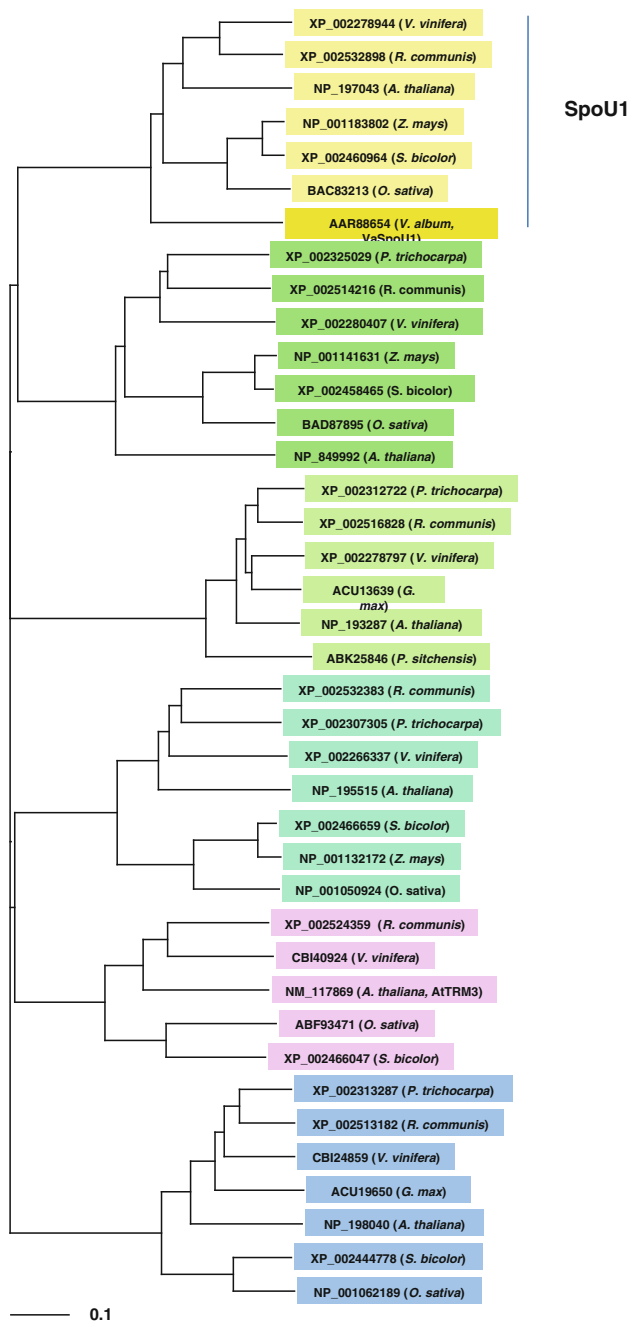
Fig. 3 Phylogenetic analysis of SpoU family proteins in plants. *Bar* indicates 10% estimated phylogenetic divergence

Fig. 4 Expression of *VaSpoU1* in vegetative tissues. *VaSpoU1* expression was determined by real time RT-PCR. The value of YL served as a control to calculate fold-induction. Data are expressed as mean ± standard deviation ($n = 3$). *YL* Young leaves, *ML* mature leaves, *ST* stems

the SpoU1 group, two subgroups were also identified; *Z. mays*, *S. bicolor*, and *O. sativa* displayed an evolutionary relationship and *V. vinifera*, *A. thaliana*, and *R. communis* were grouped separately. VaSpoU1 formed a branch of its own. This phylogenetic analysis reflects the evolutionary history of plant SpoUs among different plant species.

The subcellular localization of plant SpoU1 proteins were predicted using PSORT and WoLF PSORT programs. Interestingly, dual localization of these protein precursors to both chloroplasts and mitochondria was predicted (data not shown). Similar to these results, cleavage sites for the mitochondria presequence were also found in all SpoU1s (data not shown).

Expression and subcellular localization of VaSpoU1

To determine the expression of *VaSpoU1* in vegetative tissues, real-time RT-PCR analysis was conducted. *VaSpoU1* transcripts were detected in the stems and young and old leaves (Fig. 4). Similar levels of VaSpoU1 transcripts were found in young leaves and stems, but low levels of *VaSpoU1* were detected in old leaves as compared to other tissues.

To examine the subcellular localization of VaSpoU1, two different constructs were generated. The 1F construct contained the N-terminal region (1–120 aa) of VaSpoU1 (Fig. 5a), whereas, the putative presequence (1–24 aa) was deleted in the 2F construct. The VaSpoU1-GFP fusion constructs were introduced into *Arabidopsis* protoplasts by PEG-mediated transformation. VaSpoU1 was localized to both the chloroplasts and mitochondria (Fig. 5b). Interestingly, elimination of the putative presequence interrupted mitochondrial localization of VaSpoU1, but did not alter its localization to chloroplasts.

putative plant SpoU1s, homologous to the 368-residue VaSpoU1 precursor protein, were found that varied from 327 to 372 amino acids in length (Fig. 2). All the plant SpoU1s exhibited highly-conserved sequences similar to VaSpoU1.

As shown in Fig. 3, phylogenetic analysis was performed to identify plant SpoU family members. Six different groups of plant SpoU family members were identified, which contained a SpoU methylase domain. In
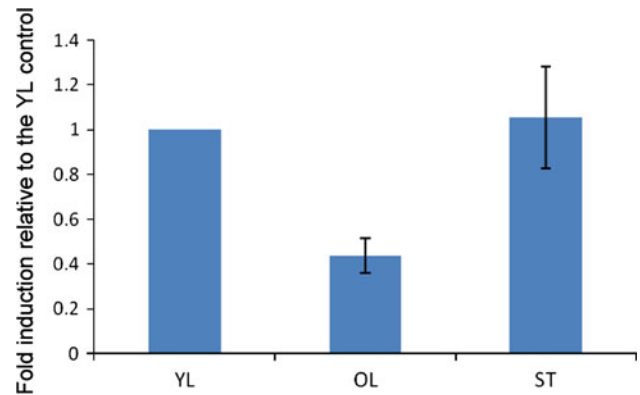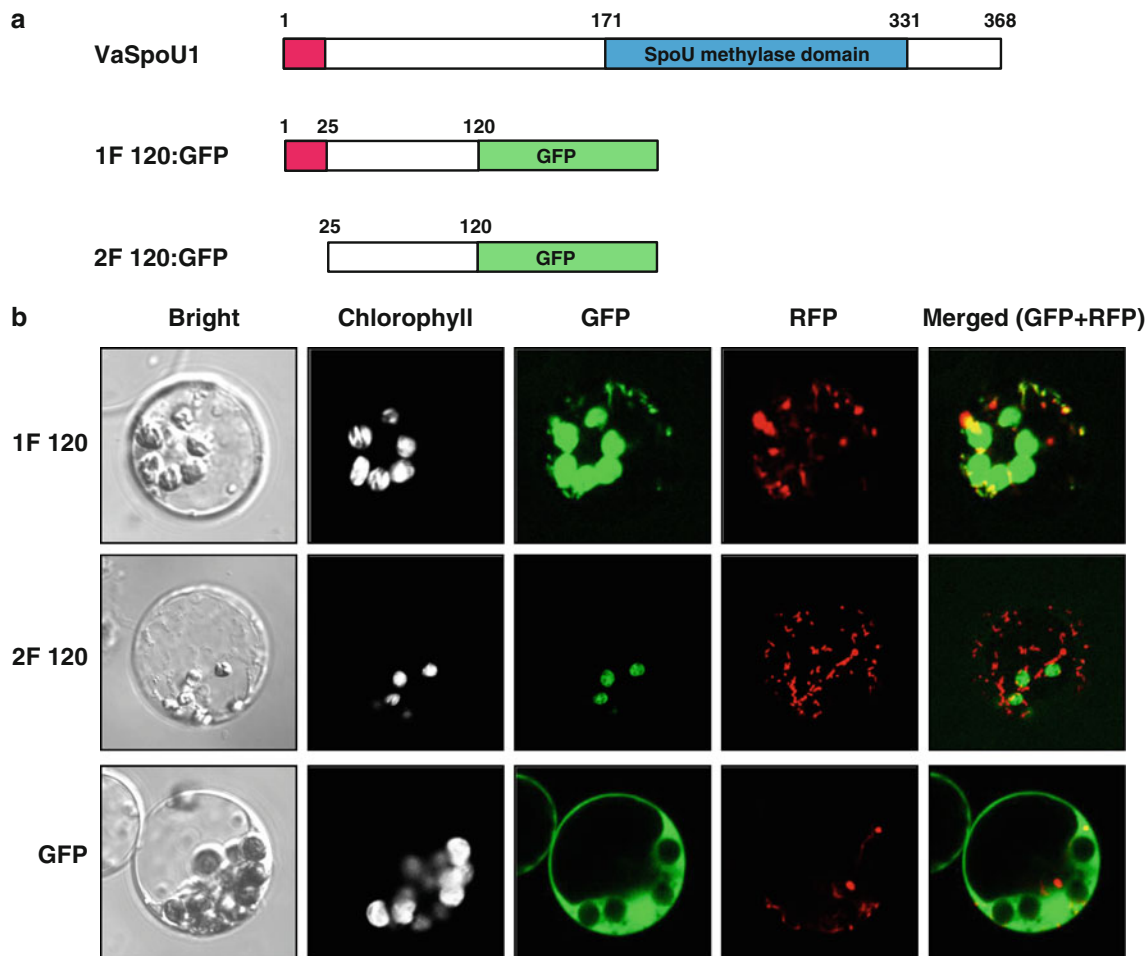
Fig. 5 Subcellular localization of VaSpoU1 in *Arabidopsis* protoplast. **a** Schematic diagrams of GFP fusion constructs. **b** Detection of VaSpoU1-GFP fusion protein in *Arabidopsis* protoplast. The mitotracker fused with RFP served as a marker for mitochondrial localization

Discussion

tRNA and rRNA methylation may affect the three-dimensional folding of RNA to regulate ligand interactions, translational fidelity, and assembling of the ribosomal subunits (King et al. 2003; Lane et al. 1995; Pintard et al. 2002; Sirum-Connolly et al. 1995). The SpoU family of proteins catalyzes 2′-*O* methylation of tRNA and rRNA (Bateman et al. 2004; Persson et al. 1997; Sirum-Connolly and Mason 1993; Thompson et al. 1982). In this study, we characterized VaSpoU1 and proposed a candidate protein for ribose 2′-*O* methylation modification of tRNA and rRNA in plant chloroplasts and mitochondria. The amino acid sequences of VaSpoU1 and other plant SpoU1s display common motifs (motifs 1–3) that are conserved in *E. coli* and yeast SpoU proteins, including TrmH, RlmB, and Pet56 (Figs. 1 and 2). Therefore, the conservation of plant SpoU1 motifs suggest that these putative methyltransferases are associated with the methylation of rRNA and tRNA.

PSORT sequence analysis predicted that all plant SpoU1s localize to chloroplasts and mitochondria (data not shown). A previous study reported that Arg residues near the cleavage site of mitochondrial presequences are conserved (von Heijne et al. 1989). An Arg residue at the −2 position of the cleavage site was detected in all plant SpoU1s (data not shown), supporting the mitochondrial localization of plant SpoU1s. Consistently, experimental subcellular localization of VaSpoU1 resulted in its localization to both mitochondria and chloroplasts. Deletion of the putative VaSpoU1 presequence inhibited localization to mitochondria (Fig. 5), indicating that the N-terminal region of VaSpoU1 may act as a mitochondrial localization signal. Therefore, plant SpoU1s may localize to both chloroplasts and mitochondria.

RNA ribose 2′-*O*-methyltransferases share common motifs and are classified as the SpoU family (Gustafsson et al. 1996; Hori et al. 2003; Watanabe et al. 2005, 2006). All enzymes characterized as SpoU family members are involved in the methylation of rRNA and/or tRNA

(Sirum-Connolly and Mason 1993; Thompson et al. 1982). For example, 2′-O-methylation at position 2251 (Gm2251) is mediated by RlmB in *E. coli* 23S rRNA (Lovgren and Wikstrom 2001). In *S. cerevisiae*, Pet56 (orthologous to RlmB) catalyzes 2′-O-methylation at position 2270 (Gm2770) of mitochondrial 21S rRNA. The *pet56*-defective mutant had insufficient mitochondrial large ribosomal subunits, suggesting that pet56 is involved in the maturation of mitochondrial 21S rRNA. TrmH, a SpoU family member, catalyzes guanosine (G18) in *E. coli* tRNAs (Persson et al. 1997). In plants, the genes responsible for G18 modification have not yet been identified. However, organelle tRNA G18 modification has been previously characterized (Francis and Dudock 1982; Marechal-Drouard et al. 1990; Marechal et al. 1985; Pirtle et al. 1981; Rozenski et al. 1999). Taking into consideration previous results and the organelle-localization data for VaSpoU1 (Fig. 5), plant SpoU1s likely participate in tRNA and/or rRNA methylation in both chloroplasts and mitochondria.

In conclusion, VaSpoU1 and other plant SpoU1s possess motifs conserved with bacterial and yeast SpoU proteins. Sequence analysis predicted SpoU1 subcellular localization to chloroplasts and mitochondria. Consistently, VaSpoU1 was experimentally detected in both chloroplasts and mitochondria. These results suggest that VaSpoU1 is associated with tRNA and/or rRNA modifications in plant organelles. Therefore, this study may offer evidence of RNA modification mediated by VaSpoU1 in plant organelles and be useful for identifying target RNAs of plant SpoU1.

References

Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, Khanna A, Marshall M, Moxon S, Sonnhammer EL, Studholme DJ, Yeats C, Eddy SR (2004) The Pfam protein families database. Nucleic Acids Res 32:D138–D141

Cavaille J, Chetouani F, Bachellerie JP (1999) The yeast *Saccharomyces cerevisiae* YDL112w ORF encodes the putative 2′-O-ribose methyltransferase catalyzing the formation of Gm18 in tRNAs. RNA 5:66–81

Chen P, Jager G, Zheng B (2010) Transfer RNA modifications and genes for modifying enzymes in *Arabidopsis thaliana*. BMC Plant Biol 10. doi:10.1186/1471-2229-10-201

Francis MA, Dudock BS (1982) Nucleotide sequence of a spinach chloroplast isoleucine tRNA. J Biol Chem 257:11195–11198

Gustafsson C, Reid R, Greene PJ, Santi DV (1996) Identification of new RNA modifying enzymes by iterative genome search using known modifying enzymes as probes. Nucleic Acids Res 24:3756–3762

Hori H, Suzuki T, Sugawara K, Inoue Y, Shibata T, Kuramitsu S, Yokoyama S, Oshima T, Watanabe K (2002) Identification and characterization of tRNA (Gm18) methyltransferase from *Thermus thermophilus* HB8: domain structure and conserved amino acid sequence motifs. Genes Cells 7:259–272

Hori H, Kubota S, Watanabe K, Kim JM, Ogasawara T, Sawasaki T, Endo Y (2003) *Aquifex aeolicus* tRNA (Gm18) methyltransferase has unique substrate specificity. TRNA recognition mechanism of the enzyme. J Biol Chem 278:25081–25090

King TH, Liu B, McCully RR, Fournier MJ (2003) Ribosome structure and activity are altered in cells lacking snoRNPs that form pseudouridines in the peptidyl transferase center. Mol Cell 11:425–435

Lane BG, Ofengand J, Gray MW (1995) Pseudouridine and O2′-methylated nucleosides. Significance of their selective occurrence in rRNA domains that function in ribosome-catalyzed synthesis of the peptide bonds in proteins. Biochimie 77:7–15

Lee YJ, Kim DH, Kim YW, Hwang I (2001) Identification of a signal that distinguishes between the chloroplast outer envelope membrane and the endomembrane system in vivo. Plant Cell 13:2175–2190

Lovgren JM, Wikstrom PM (2001) The *rlmB* gene is essential for formation of Gm2251 in 23S rRNA but not for ribosome maturation in *Escherichia coli*. J Bacteriol 183:6957–6960

Marechal L, Guillemaut P, Grienenberger JM, Jeannin G, Weil JH (1985) Sequence and codon recognition of bean mitochondria and chloroplast tRNAsTrp: evidence for a high degree of homology. Nucleic Acids Res 13:4411–4416

Marechal-Drouard L, Neuburger M, Guillemaut P, Douce R, Weil JH, Dietrich A (1990) A nuclear-encoded potato (*Solanum tuberosum*) mitochondrial tRNA(Leu) and its cytosolic counterpart have identical nucleotide sequences. FEBS Lett 262:170–172

Persson BC, Jager G, Gustafsson C (1997) The spoU gene of *Escherichia coli*, the fourth gene of the spoT operon, is essential for tRNA (Gm18) 2′-O-methyltransferase activity. Nucleic Acids Res 25:4093–4097

Pintard L, Bujnicki JM, Lapeyre B, Bonnerot C (2002) MRM2 encodes a novel yeast mitochondrial 21S rRNA methyltransferase. EMBO J 21:1139–1147

Pirtle R, Calagan J, Pirtle I, Kashdan M, Vreman H, Dudock B (1981) The nucleotide sequence of spinach chloroplast methionine elongator tRNA. Nucleic Acids Res 9:183–188

Rozenski J, Crain PF, McCloskey JA (1999) The RNA modification database: 1999 update. Nucleic Acids Res 27:196–197

Sirum-Connolly K, Mason TL (1993) Functional requirement of a site-specific ribose methylation in ribosomal RNA. Science 262:1886–1889

Sirum-Connolly K, Peltier JM, Crain PF, McCloskey JA, Mason TL (1995) Implications of a functional large ribosomal RNA with only three modified nucleotides. Biochimie 77:30–39

Thompson J, Schmidt F, Cundliffe E (1982) Site of action of a ribosomal RNA methylase conferring resistance to thiostrepton. J Biol Chem 257:7915–7917

von Heijne G, Steppuhn J, Herrmann RG (1989) Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem 180:535–545

Watanabe K, Nureki O, Fukai S, Ishii R, Okamoto H, Yokoyama S, Endo Y, Hori H (2005) Roles of conserved amino acid sequence motifs in the SpoU (TrmH) RNA methyltransferase family. J Biol Chem 280:10368–10377

Watanabe K, Nureki O, Fukai S, Endo Y, Hori H (2006) Functional categorization of the conserved basic amino acid residues in TrmH (tRNA (Gm18) methyltransferase) enzymes. J Biol Chem 281:34630–34639