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A *bona fide* La protein is required for embryogenesis in *Arabidopsis thaliana*

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ABSTRACT

Searches in the *Arabidopsis thaliana* genome using the La motif as query revealed the presence of eight La or La-like proteins. Using structural and phylogenetic criteria, we identified two putative genuine La proteins (At32 and At79) and showed that both are expressed throughout plant development but at different levels and under different regulatory conditions. At32, but not At79, restores *Saccharomyces cerevisiae* La nuclear functions in non-coding RNAs biogenesis and is able to bind to plant 3′-UUU-OH RNAs. We conclude that these La nuclear functions are conserved in *Arabidopsis* and supported by At32, which we renamed as AtLa1. Consistently, AtLa1 is predominantly localized to the plant nucleoplasm and was also detected in the nucleolar cavity. The inactivation of AtLa1 in *Arabidopsis* leads to an embryonic-lethal phenotype with deficient embryos arrested at early globular stage of development. In addition, mutant embryonic cells display a nucleolar hypertrophy suggesting that AtLa1 is required for normal ribosome biogenesis. The identification of two distantly related proteins with all structural characteristics of genuine La proteins suggests that these factors evolved to a certain level of specialization in plants. This unprecedented situation provides a unique opportunity to dissect the very different aspects of this crucial cellular activity.

INTRODUCTION

The La protein is a highly abundant phosphoprotein first described in human as an autoantigen in patients suffering from the rheumatic diseases, systemic lupus erythematosus and Sjögren's syndrome (1,2). It is an RNA-binding protein involved in many aspects of RNA metabolism (3–5) and is present in a wide range of eukaryotes including budding and fission yeasts, vertebrates, insects, worm (5) and trypanosome (6). The La protein is one of the first proteins to bind to primary polymerase III (pol III) transcripts due to the specific recognition of the 3′-UUU-OH motif present in these precursors (7). The *Saccharomyces cerevisiae* La protein (named Lhp1p) also binds polymerase II (pol II) transcribed small RNAs that terminate in 3′-UUU-OH such as precursors to the U3 snoRNA (small nucleolar RNA) or U snRNAs (small nuclear RNA) (8–10). From yeast to human, genetic and biochemical studies have shown that La protects these small RNAs from 3′–5′ exonucleases (5,11–13). For example, the binding of La to pre-tRNA precursors prevents exonucleolytic nibbling of their 3′-trailer and promotes its endonucleolytic removal (14–17) and Lhp1p stabilizes U snRNAs and U3 precursors from exonucleolytic degradation (9,10,18). In addition, the La protein fulfils an RNA chaperone activity (19) involved in the assembly of several RNPs (10,18) and in the structural stabilization of pre-tRNAs (20,21). Finally, the La protein most probably takes part in the quality-control mechanism of newly synthesized non-coding RNAs such as pre-tRNAs (17,20,22).

To accomplish its various functions in the biosynthesis of small stable RNAs, most of the La protein logically accumulates in the nucleoplasm as assessed by steady-state subcellular localization experiments (23–25). Nevertheless, the La protein subcellular distribution is highly dynamic as this protein was shown to shuttle through the nucleolus in association with several precursor RNAs (3,24–26) and strongly accumulates in the nucleolus during late G1 and early S phases for yet unknown reasons (24). But not all La protein is present in the nuclear compartment. It has been demonstrated that 2–4% of the *Xenopus* La homologue accumulates in the cytoplasm (27) and that the human La (hLa) protein shuttles between nucleus and cytoplasm (28). Moreover, a major pool of La protein is redistributed to the cytoplasm under various stress conditions such as apoptosis (29) or

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viral infections (30,31). These observations are in agreement with several reports suggesting that, beside their primary role in pol III and non-coding pol II stable RNAs biosynthesis, La homologues could be implicated in mRNA translation enhancement [for review see (5)]. For example, by binding to their 5′-UTR, the cytoplasmic La protein stimulates the internal ribosome entry site-mediated translation of viral mRNAs (32,33) as well as certain cellular mRNAs (34,35). Also, La is involved in the cap-dependent translation of 5′-terminal oligopyrimidine stretch (TOP) containing mRNAs (27).

La proteins are modular polypeptides whose molecular weight ranges from ~50 kDa in vertebrates to 32 kDa in yeasts. Their NH2-terminal domain (NTD) is extremely well conserved and always contains a 60–80-amino-acid-domain called the La-motif, also found in a number of otherwise unrelated (La-like) proteins (5), closely followed by a canonical RNA recognition motif (RRM1) (36). Diverse structural (37–40) and functional studies (4,5) emphasized the importance for a *bona fide* La protein to display this particular NTD organization at least for efficient and specific binding of 3′-UUU-OH-containing substrates. The COOH-terminal domain (CTD) of La proteins is more variable. The hLa CTD contains an atypical RRM (RRM2) ending with a long helix comprising a nuclear retention element (41,42), followed by a short basic motif (SBM), several phosphorylation sites (23), a nucleolar localization signal (NoLS) (25) and a nuclear localization signal (NLS). The RRM2 motif is found in La proteins from all vertebrates, but is absent from the very short CTD of the yeast proteins and was not detected for La homologues from some metazoans such as fly or worm (5).

In higher plants, a functional homologue of the La protein is yet to be identified. We report here that higher plants are exceptional compared to other eukaryotes by having two distantly related proteins that display every structural feature of genuine La proteins. We show that at least one of these proteins we named AtLa1 (for *Arabidopsis thaliana* La protein 1) is a true functional homologue as it is able to fulfil the nuclear La functions related to RNA pol III transcripts maturation and stability. We also demonstrate that T-DNA insertion in the *AtLa1* gene leads to embryonic lethality showing that AtLa1 function is required for plant viability.

MATERIALS AND METHODS

Molecular phylogenetic analyses

The amino acid sequences of the different La motifs were aligned using ClustalW multiple-alignment program (43). Evolutionary distances were calculated using the probability matrix from blocks (PMB) model (44) of the Protdist program (PHYLIP package version 3.6, available at the following web site: http://evolution.genetics.washington.edu/phylip.html). The coefficient of variation of the gamma distribution (to incorporate rate heterogeneity) was obtained by pre-analysing the data with the Tree-Puzzle program (45), and the significance of the various phylogenetic lineages was assessed by bootstrap analyses (1000 trials) (46). The phylogenetic tree and the consensus tree were inferred using the Neighbour-Joining (NJ) and Consense programs (both from the PHYLIP package), respectively.

Cloning

cDNAs corresponding to At32 and At79 isoforms were PCR amplified from a cDNA library derived from Col0 suspension cell line (47). PCR products were inserted in pBluescript vector and two clones for each cDNA were fully sequenced. Plasmids were named p116 (At32 in pBSK) and p114 (At79 in pBSK), respectively. The centromeric yeast shuttle vector pFL38 (*ARSCEN-URA3*) (48) was used as a platform to assemble the different genes under the control of the *GAL1-10* promoter region (49) with or without the Protein A tag (50). First, a 1169-bp SacI-BamHI and a 264-bp BamHI-HindIII restriction fragments, both from the pGALPATG1L vector (obtained from K. Hellmut and E. Hurt, University of Heidelberg), containing the GAL1-10 promoter region fused with the Protein A tag, and the GAL4 transcription terminator region, respectively, were cloned into pFL38 to produce the pFL38GALProtA vector (p131). The *Arabidopsis* open reading frames (ORFs) were PCR amplified from plasmids p116 and p114, respectively, while the *LHP1* gene was amplified from the pATL vector [generous gift from Sandra Wolin (HHMI, New Haven)]. All PCR amplifications were performed using a 5′-primer with a terminal BamHI site (to produce the tagged version) or with a terminal SphI site (to produce the untagged version) in combination with a 3′-primer ending with an XhoI site. The tagged and untagged final constructs were produced by cloning the different PCR products in pFL38GALProtA after digestions with BamHI-XhoI (tagged version) or with an SphI-XhoI (untagged version). By this way, we obtained the pGAL [numbers p140 (*LHP1*), p139 (At32) and p138 (At79)] and pGALPa [numbers p133 (*LHP1*), p132 (At32) and p135 (At79)] collections. To construct the high-copy vectors, the *ARSCEN* regions of plasmids p131, p133 and p132 were excised by ClaI digestion and replaced with the 2 μL region of pFL44L (48) to produce the p2μGALPa plasmids [p144 (Protein A alone), p150 (*LHP1*), p145 (At32) and p146 (At79)].

Restriction sites (NheI-SmaI at the 5′-end and BamHI-XbaI at the 3′-end) were added by PCR amplification to the eGFP ORF. The PCR amplification product was inserted into pBluescript vector at sites HincII and XbaI to obtain pBSK-eGFP (p315) and verified by sequencing. The At32-coding region was subsequently inserted into the BamHI and XbaI sites of the pBSK-eGFP resulting in NH2 GFP fusion (vector p316), and into the XhoI and NheI sites to give COOH fusion (vector p317). After sequencing, each fusion as well as the eGFP ORF were transferred at the KpnI and XbaI restriction sites of the plant transformation vector pBIN-HYG-TX (51) under the control of the cauliflower mosaic virus 35S promoter (CaMV35S) giving plasmids p320 (GFP), p321 and p322 (GFP-At32 and At32-GFP).

Yeast strains and media

Growth and handling of *S. cerevisiae* were by standard techniques (52). The red/white sectoring medium contained 1% bactopeptone, 0.5% yeast extract and 4% galactose. Fluoroorotate tests were performed on Yeast Nitrogen Base (YNB) medium containing 2% galactose, supplemented as required in amino acids and containing 1mg/ml of proline and 1 mg/ml of fluoroorotic acid (Melford Lab. Ltd, ref F5001). The BP1 (*Mat*a *lsm8-1 ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*/pATL), CY2 (*Mat*α *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*) and CY3 (*Mat*α *ura3 lys2 ade2 trp1 his3 leu2*) strains (16,53) used in the present work were kindly provided by Sandra Wolin (HHMI, New Haven). Yeast transformations were performed as described (54) except that 6% DMSO was added prior to heat shock and the final pellet was resuspended in 0.15 M NaCl.

Yeast red/white sectoring and fluoroorotic acid assays

The BP1 strain was transformed with the different sets of yeast plasmids and plated on minimal medium lacking uracil and containing 2% glucose. The sectoring assays were always performed as follow, with two transformants for each plasmid previously selected at least twice on YNB plates lacking uracil and containing 2% galactose. The transformants to be tested were grown overnight at 30°C in liquid minimal medium lacking uracil and containing 2% galactose until cultures reached 1 $OD^{600\,nm}$. They were then diluted in the same medium and monitored for growth until they reached 0.5 $OD^{600\,nm}$. Cells were then plated on sectoring medium at 200–500 cells per plate. After 4 days growth at 30°C, the percentage of red/white sectoring was determined. For further tests, red sectors were streaked on rich galactose medium as many times as necessary to give only solid red colonies. An independent red clone was then tested on appropriate plates for auxotrophy to tryptophan and uracil and for its ability to grow on fluoroorotic-acid-containing plates.

Yeast RNA analysis

The CY2 strain was transformed with the p2μGALPa plasmids and plated on minimal medium lacking uracil and containing 2% glucose. Two transformants for each plasmid were streaked at least twice on YNB plates lacking uracil and containing 2% galactose. Cultures were then conducted as for the sectoring assays and total RNAs were extracted as described (55). To analyse tRNAs and U3 snoRNA, 5 μg of total RNAs were fractionated on polyacrylamide 8.3 M urea gels. RNAs were then blotted on nitrocellulose Hybond N+ membranes (Amersham Biosciences). Hybridizations were performed as described using previously published oligonucleotide probes (9,56).

Yeast western blot analysis

Total protein extracts were prepared as described (57) from 5 $OD^{600\,nm}$ cell cultures prepared for sectoring assays or northern blot analysis. Proteins were fractionated on 10% SDS-PAGE gels and blotted on nitrocellulose membrane with Trans-Blot semi-dry system (BioRad). The blots were reacted with rabbit anti-Nhp2p [generous gift from M. Caizergues-Ferrer (LBME, Toulouse)] (58) at 1/5000 dilution as primary antibody and donkey anti-rabbit IgGs horse radish peroxydase linked (Amersham Biosciences) at 1/10 000 dilution as secondary antibody.

Antisera production and plant western blot analysis

Antibodies were produced using the Eurogentec double X immunization programme followed by affinity-column purifications. Briefly for each protein, rabbits were inoculated with a mix of two synthetic peptides (pep1 and pep2) corresponding to specific regions of the protein. For both proteins, pep1 is located in the central region between RRM1 and RRM2 (At32pep1: H_2N-CQPQKGSANQKNGSDH-$CONH_2$, At79pep1: H_2N-CLGKSESHNEFRRGQI-$CONH_2$,) and pep2 corresponds to the very last 16 or 15 amino acids of the proteins (At32pep2: H_2N-CDSPGGRWNKSQK VEA-COOH, At79pep2: H_2N-CFENVQPTKKARKEP-COOH). Sera from rabbit's final bleeding were divided into two and each sample was affinity purified against pep1 or pep2, respectively. To assess the immunogenicity of each purified serum, we performed western blot analysis on total extracts prepared from yeasts expressing At32 or At79, wild-type *Arabidopsis* cell suspension or 2-weeks-old seedlings. For both At32 and At79, only the sera fraction purified against pep2 gave a satisfying immunogenic response and was used in subsequent western blot analyses.

To prepare total protein extracts, plant materials (plant tissue or cell suspension) were suspended in Laemmli sample buffer supplemented with 0.2 M DTT and 10% Protease Inhibitor Cocktail (P9599 Sigma) in a 1.5-ml microtube and crushed with a small glass pestle and sea sand. Extracts were incubated for 5 min at 95°C and cleared by centrifugation. Gel separation and blotting were performed as described above in the yeast western blot section.

Arabidopsis plant and cell suspension material

The T-DNA insertion lines SAIL 548H11 (59) and GABI 870F12 (60) were obtained from the ABRC Stock Centre and the Max Planck Institute for Plant Breeding Research, respectively.

To prepare transgenic cell suspension lines expressing the different GFP constructs, *Arabidopsis* T87 cells were transformed with the different plant transformation plasmids using *Agrobacterium tumefasciens*, as previously described (61). For each construct, cell lines were screened by western blot analysis with anti-GFP monoclonal antibodies (Clontech) for expression of the transgenic protein. Cell lines were maintained under constant hygromycine selection (25 μg/ml) as liquid suspensions and as calluses growing on solid media, as previously described (61).

Preparation of crude cell extracts and immunoprecipitation experiments

Here, 2 g of *Arabidopsis* cell suspension expressing GFP fusions, obtained by filtration of 3–4-day-old liquid culture is resuspended in 6 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 2.5 mM $MgCl_2$, 0.1% Triton 100×) with 1 mM DTT, 10 mM vanadyl ribonucleoside complex (Biolabs), 1% protease inhibitor cocktail (P9599 Sigma), 2 mM benzamidine, 1 mM phenyl methyl sulphonyl fluoride and 10 μM decarboxylase inhibitor. The suspension is loaded in the pre-cooled chamber of a 'one-shot' cell disrupter system (Constant Systems Ltd), and cells are lysed under a pressure of 552 bar. The extract is subsequently cleared by centrifugation (30 min, 4200 *g*, 4°C).

For immunoprecipitation, crude extracts were incubated with a 1/300 dilution of anti-GFP full-length polyclonal antibody (Clontech) on a rotary shaker for 3 h at 4°C and then mixed with magnetic nanoparticles conjugated with Protein A (Bio-Adembeads Protein A, Ademtech S.A) and incubated for an additional hour. After three washes with lysis buffer, beads were divided as follows: one-ninth of the beads was resuspended in Laemmli buffer and heated 5 min at 95°C for protein analysis and the remaining beads were eluted with urea 8 M 5 mM EDTA buffer and extracted once with phenol:chloroform:isoamyl alcohol. RNAs were then precipitated by adding 40 μg of glycogen and 2.5 volumes of absolute ethanol and resuspended in RNase-free water. To compare the different transgenic lines, equivalent fractions of proteins or RNAs were used for western blot or RT-PCR analysis. Proteins were analysed by western blot with an anti-GFP monoclonal antibody (Clontech). Chemiluminescent signal was quantified using the VersadocImaging system (BioRad). RNAs were treated with the DNA-free kit from Ambion following the manufacturer's 'Rigorous DNase treatment' protocol. A fraction of the RNA (usually one-fifth) was reverse transcribed with expand reverse transcriptase and hexanucleotides as primer (Roche). cDNAs were then PCR amplified with primer pairs specific to pre-$tRNA^{Met}$, pre-tsnoR43.1 or 5.8S RNA.

Confocal microscopy

Protoplasts were prepared from cell suspensions expressing the GFP fusions as described by Sheen,J. (2002, A transient expression assay using *Arabidopsis* mesophyll protoplasts. http://genetics.mgh.harvard.edu/sheenweb) using an overnight digestion in the dark. Images were obtained with a Zeiss Laser Scanning Microscope LSM 510.

Study of embryonic phenotypes

Wild-type and mutant seeds were collected from hemizygous siliques at different stages of maturity. The seeds were fixed in ethanol:acetic acid (3:1) for 20 min, followed by a slow rehydration in a series of ethanol–water solutions. After rehydration, the seeds were cleared in Hoyer's solution (2.5 g gum arabic, 100 g chloralhydrate, 5 ml glycerol in 30 ml of H_2O) and observed using Nomarski optics with a Zeiss Axioskop2 microscope (Carl Zeiss, Germany).

RESULTS

Identification of La and La-like proteins in *Arabidopsis thaliana* and *Oryza sativa*

We searched (by a protein BLAST at http://www.ncbi.nlm.nih.gov) the *A. thaliana*- and rice (*Oryza sativa*)-expressed genomes for proteins presenting similarities with the eukaryote consensus La-motif (SMART accession number SM00715, http://smart.embl-heidelberg.de). Our search revealed the presence of eight *Arabidopsis* and nine rice putative proteins containing a La motif (Figure 1). We used a phylogenetic approach to test the relationship of *Arabidopsis* and rice La motifs with those present in several eukaryote La and La-like proteins (Figure 1A). Our results show that two *Arabidopsis* (At4g32720 and At1g79880) and two rice (bad19607 and cae03115) proteins form a well-supported cluster (bootstrap of 1000) and that the association of this cluster with the *Schizosaccharomyces pombe* and *S. cerevisiae* La proteins is fairly well supported (bootstrap of 728, Figure 1A). The NTD of La proteins contains a typical RRM (SMART accession number SM00715) (referred to as RRM1) that closely follows the La motif. In addition to At4g32720, At1g79880, bad19607 and cae03115, three *Arabidopsis* and four rice proteins also present a RRM closely following the La motif (Figure 1B), but these proteins group in a cluster intermediate between genuine La and La-like proteins (Figure 1A). The use of the first RRM, instead of the La motif in the phylogenetic analysis also clearly distinguishes these proteins from *bona fide* La homologues (not shown).

The CTD of the hLa protein possesses a novel type of RRM (called RRM2), the structure of which is atypical compared to canonical RRMs (42). This motif is very difficult to detect based on primary sequence analysis alone. We hence searched for the presence of such atypical RRMs in the plant putative La homologues using the hidden-Markov-model (HMM)-based protein structure prediction program SAM-T02 (http://www.cse.ucsc.edu/research/compbio/HMM-apps/T02-query.html). This program was successful in predicting the correct topology for the human atypical RRM2 (see Supplementary Figure 1 online). We found that amongst the plant proteins displaying both the La motif and the canonical RRM, At4g32720 and At1g79880 from *Arabidopsis* and bad19607 and cae03115 from rice are the only ones likely to possess an atypical RRM2 domain in their CTDs (Figure 1B and Supplementary Figure 1 online). We also found that with the exception of the *S. pombe* and *S. cerevisiae* homologues, each of the genuine La proteins we tested is likely to display such motif (Figure 1B, Supplementary Data Figure 1 online). Therefore, the presence of an atypical RRM in the La protein CTDs is apparently not a characteristic restricted to vertebrate proteins, but is likely to be a conserved feature of genuine La proteins with the exception of the yeast homologues,

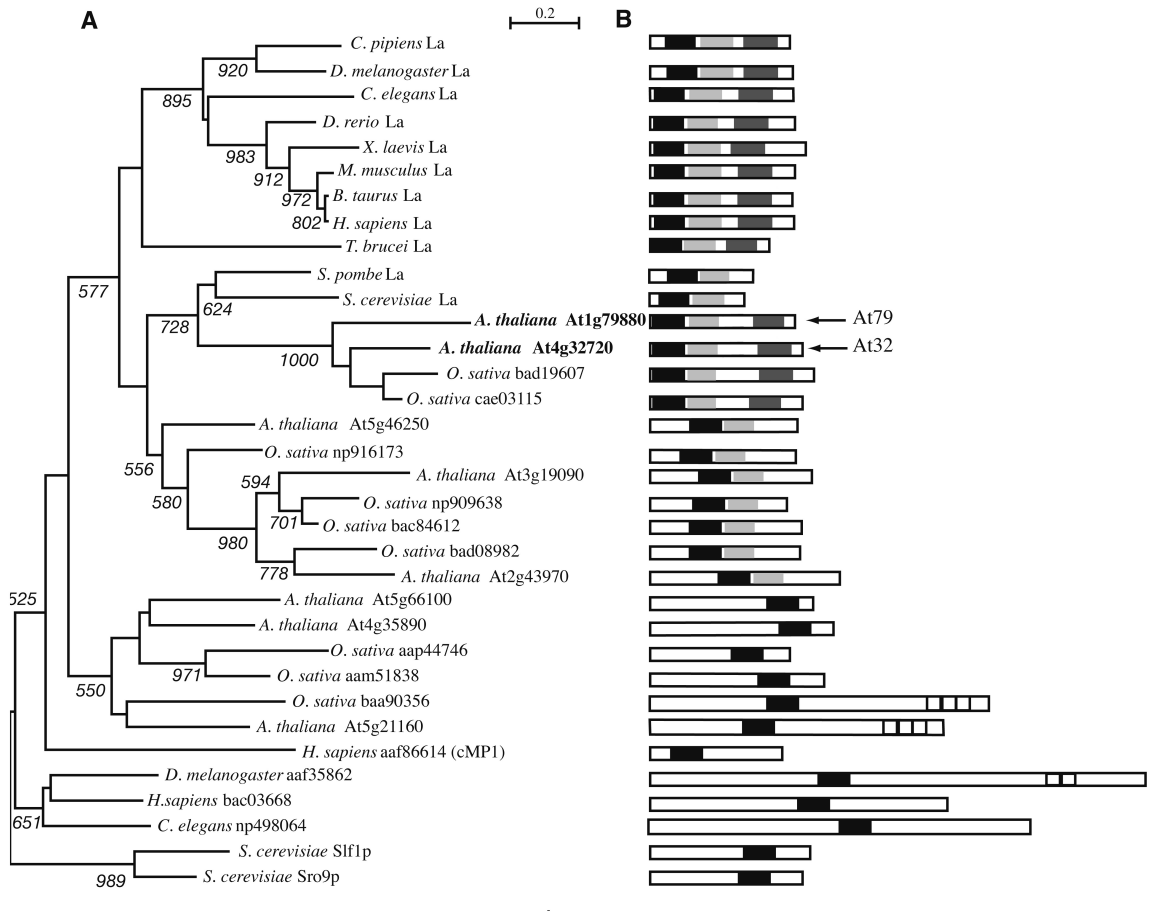


Figure 1. Comparison of La and La-like proteins from various eukaryotes. (**A**) Neighbour-Joining tree representing the phylogenetic relations among La motif-containing proteins. Numbers above each node indicate bootstrap values out of 1000 replicates (only values over 500 are presented). The amino acids divergence scale is indicated. The names of the proteins selected for further analysis are in bold. (**B**) Structural comparison of the different La domain-containing proteins. La domains are represented by black boxes, the first RRM domains by light grey boxes, the atypical second RRM by dark grey boxes and the DM15 motifs (a motif of unknown function) by open boxes. Arrows to the right respectively point to A1g79880 (At79) and At4g32720 (At32).

which possess a much shorter CTD. Altogether these data suggest that *Arabidopsis* At4g32720 and At1g79880 and rice bad19607 and cae03115 are the most likely *bona fide* La homologues of the two species.

The two *Arabidopsis* proteins, At4g32720 and At1g79880, have an overall amino acid identity of only 44%, with the highest identity found in the La motif (54%) and in the first RRM (59%) (see Supplementary Data Figure 1A online), suggesting that they are not produced from recently duplicated genes. In fact our phylogenetic studies show that At4g32720 is more closely related to the two rice proteins than it is to At1g79880. The presence of two distantly related proteins in *Arabidopsis* with characteristics of *bona fide* La proteins is exceptional as the eukaryote La function has always been associated to a single La protein [with the exception of two closely related (>90% amino acids identity) *Xenopus* La proteins (62)].

Expression of At4g32720 and At1g79880 in *Arabidopsis*

Searches of the *Arabidopsis* information resources (TAIR at http://www.arabidopsis.org) and NCBI databases revealed that full-length cDNAs corresponding to both loci have been sequenced. For the At4g32720 locus, all characterized ESTs and full-length cDNAs code for a single protein reported as At4g32720.1. For the At1g79880 locus, three different isoforms are suggested by EST data but only two of those (called At1g79880.1 and At1g79880.2) are supported by full-length cDNAs. The At1g79880.2 protein differs from At1g79880.1 by the deletion of the first 44 amino acids, including more than half of the La motif. Since At1g79880.2 lacks a complete La motif and since the third isoform (At1g79880.3) is only supported by a single EST sequence (corresponding to a small portion of the protein CTD), we decided to conduct further structural and functional studies on the At1g79880.1 protein. From now on, the At4g32720.1 and At1g79880.1 proteins will be referred to as At32 and At79, respectively.

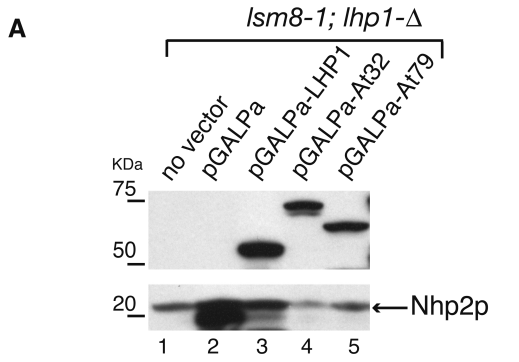
To monitor the developmental expression profiles of At32 and At79, we searched the expression atlas of *Arabidopsis* development microarray data (AtGenExpress) using the Genevestigator web site (https://www.genevestigator.ethz.ch/at/) (63). We observed that mRNAs

corresponding to both genes are present at all developmental stages in every plant tissue tested (see Supplementary Figure 2A online). At32 mRNA levels are highest in tissues composed of actively dividing cells (such as root tips, radicles, seedling, callus or cell suspensions), while At79 mRNAs is in general more abundant in tissues composed of mainly differentiated (non-dividing) cells. In average, At32 mRNAs are four times (4×) more abundant than that of At79 but this ratio is highly variable and is highest (10×) in 'young' tissues and lowest (2×) in 35-day-old senescent and cauline leaves (see Supplementary Figure 2B online). Western blot analysis using antibodies specific for At32 and At79 confirmed that both proteins are present in all developmental stages tested (see Supplementary Figure 2C online).

To further characterize the roles of At32 and At79, we first conducted functional studies in yeast and tested the ability of these plant proteins to complement phenotypes linked to the full depletion of Lhp1p, the *S. cerevisiae* La homologue (16).

At32 restores growth of the *lsm8-1;lhp1-Δ* colethal strain

While Lhp1p is not required for growth in wild-type cells, it becomes essential in specific genetic backgrounds (10,18,21,53). The Sm-like protein 8 (Lsm8p) is an essential member of the Lsm2p–Lsm8p ring-shaped complex (64,65). In the nucleus, Lsm8p associates with U6 snRNA and is important for its stability, for the formation of U6-containing snRNPs and for pre-mRNA splicing (18,64,66,67). The *lsm8-1* allele is not lethal in normal growth conditions but becomes essential when combined with a deletion of the *LHP1* gene. In the *lsm8-1* background, Lhp1p is essential to stabilize newly synthesized U6 RNAs and to facilitate the U6 snRNP assembly (18). The viability of the BP1 (*lsm8-1;lhp1-Δ*, *ade2*) colethal strain is maintained by an extra-chromosomal copy of the *LHP1* gene carried on an *ARSCEN*, *ADE2*, *TRP1* plasmid (pATL). As a red pigment accumulates in *ade2* mutant strains (68), cells that retain pATL will form white colonies, while cells that grow without the plasmid will form solid red colonies (18,53). The ability of plant proteins to restore growth in the *lsm8-1;lhp1-Δ* background can be monitored by the capacity of BP1 to form colonies with red sectors when transformed with a plasmid expressing the coding sequence of interest. DNA fragments corresponding to the *LHP1*, At32 and At79 ORFs were cloned in fusion at their NH2 terminus with the Protein A tag (50) and placed under the control of a galactose-inducible promoter on a low-copy *URA3* vector (48) to create the pGALPa plasmids collection. BP1 was transformed with the different pGALPa plasmids and grown under galactose-inducing conditions. To compare protein production levels in the different transformants, proteins were prepared from an aliquot of each culture and western blot analysis performed using a rabbit anti-Nhp2p (a snoRNP H/ACA protein) antibody (58) that, as for most IgGs, also binds to the Protein A tag (50). As expected, the anti-Nhp2p antibody recognizes in all extracts a single protein migrating above 20 kDa, corresponding to Nhp2p (Figure 2A). Additional bands



B

Expressed prot. / vector	Prot A	Lhp1p	At32	At79
no vector	<0.2			
pGALPa	0.6	22.2	2.9	0.7
pGAL		23.4	1.9	0.5
p2μGALPa	<0.2	45	8	<0.2

Figure 2. Complementation of the *lsm8-1;lhp1-Δ* colethal phenotype by heterologous expression of the At32 and At79 proteins. (**A**) Expression of At32 and At79 proteins in yeast. Western blot analysis of total protein extracts from the untransformed BP1 (*lsm8-1;lhp1-Δ*) strain (lane 1) or the BP1 strain expressing Protein A (lane 2), ProtA-Lhp1p (lane 3), ProtA-At32 (lane 4) or ProtA-At79 (lane 5) from the pGALPa set of vectors using the rabbit anti-Nhp2p antibody (58). The Lhp1p has a predicted molecular weight of 32 kDa but displays an electrophoretic mobility of 38 kDa (69), accordingly the ProtA-Lhp1p migrates above 50 kDa (lane 3). The At32 protein extracted from *Arabidopsis* has an apparent molecular weight of 56 kDa (see Supplementary Figure 2C online) and consistently the fusion protein migrates at ~74 kDa (lane 4). The ProtA-At79 fusion is detected at ~62 kDa (lane 5) as expected from the At79 predicted size of 44 kDa, which was confirmed by western blot analysis of plant extracts with antibodies specific for At79 (see Supplementary Figure 2C). (**B**) Sectoring assays. The BP1 strain transformed with the three sets of plasmids was grown under galactose-inducing conditions and plated on sectoring medium. Two independent experiments were conducted using a different transformant for each plasmid in each case. A total of ~1000 colonies per plasmid were analysed in each test and the percentage of sectoring colonies determined as: [(red colonies plus colonies with at least one red sector)/(number of colonies screened)] × 100. The reported numbers correspond to the average of the percentages obtained in each experiment.

corresponding to the expression of the Protein A alone (Figure 2A, lane 2) and to the different fusion proteins (Figure 2A, lanes 3–5) were detected in extracts from the BP1 strain transformed with the different pGALPa plasmids. Using the Nhp2p signal as a loading control, we can observe that all fusion proteins accumulate to similar levels (Figure 2A).

The galactose-induced cultures of transformed and untransformed BP1 strains were plated on sectoring

medium and the number of colonies with red sectors was counted and expressed as a percentage of the total number of colonies (Figure 2B). As expected, the ProtA-Lhp1p positive control restores red/white sectoring in the BP1 strain giving 22.2% of colonies with red sectors while background, as observed for the untransformed and Protein A-expressing colethal strain, is between <0.2% and 0.6%. Among the *Arabidopsis* proteins, only At32 gives red/white sectoring above background (2.9%). Using fluoroorotic-acid-containing medium (68), we confirmed that the red colonies growth is strictly dependent on the presence of the pGALPa vectors expressing ProtA-Lhp1p or ProtA-At32. The inability of the At79-tagged protein to restore growth in the BP1 strain might be due to the presence of the tag or to the fact that a higher level of this protein is required. To test these possibilities, we placed the untagged plant and *LHP1* ORFs under the control of the *GAL* promoter on the *ARSCEN-URA3* plasmid (pGAL vectors) and the galactose controlled protA-tagged ORFs on a high-copy *2μL-URA3* plasmid (48) (p2μGALPa vectors). Each set of plasmids was transformed into the BP1 strain and tested by western for equivalent protein expression levels using anti-Nhp2p antibodies for p2μGALPa vectors and At32- and At79-specific antibodies for the pGAL vectors (not shown). As with the pGALPa plasmids collection, we tested the ability of the new BP1 transformants to produce red sectors. The untagged proteins gave results similar to the previous ones showing that the presence of the NH2 tag has no clear impact on the expressed proteins (Figure 2B). As expected, the use of high-copy vectors leads to higher levels of red sectors for ProtA-Lhp1p and ProtA-At32 giving 45 and 8% red/white sectoting colonies respectively, whereas the At79 sectoring percentages remain at the background level (Figure 2B).

Altogether these data show that the At32 putative La homologue is able to complement the colethal *lsm8-1;lhp1-Δ* phenotype and suggest that At79 is not able to do so.

At32 is able to restore 3′-end processing of non-coding RNA precursors in an *lhp1-Δ* background

Inactivation of the *LHP1* gene in an otherwise wild-type background leads to several molecular phenotypes including a differential accumulation of U3A snoRNA (referred to as U3 from now on) and tRNA precursor species (9,16). To test the ability of the plant proteins to complement molecular phenotypes observed upon Lhp1p depletion, we expressed At32 and At79 from the p2μGALPa set of vectors in the CY2 (*lhp1::LEU2*) yeast strain which bears a fully inactivated allele of the *LHP1* gene (16). The transformed as well as untransformed CY2 and the isogenic CY3 wild-type strains (69) were grown on galactose-inducing medium. We confirmed by western blot analysis the proper and equivalent expressions of the different fusion proteins in the CY2 background under these growth conditions (data not shown). Total RNAs were extracted from the different transformants and the accumulation of U3 and tRNAs precursors assessed. Three independent experiments were performed and gave similar results.

Lhp1p has been shown to protect two U3-3′ extended forms from exonucleolytic trimming by binding to poly(U) stretches present in the 3′-end region of pre-U3 molecules (Figure 3A). These extended forms, named U3-3′I (U3 + 12) and U3-3′II (U3 + 18) are readily detected by northern blot in wild-type conditions (Figure 3A lane 2 and drawing). Following *LHP1* inactivation, the two extended forms are replaced by a heterogeneous population of molecules whose sizes range from +12 to +8 (9). Consequently, the impact of *LHP1* inactivation on pre-U3 3′-maturation can be assessed by monitoring the level of the U3-3′II extended form. Our analysis shows a strong impact on the accumulation of the U3-3′II form upon inactivation of *LHP1* (Figure 3A lane 1) but by contrast to previous report (9), there is no complete depletion of the band. This might result from strain discrepancies since we are using a different genetic background (16) than the one used by Kufel *et al.* (9). In extracts from the CY2 strain transformed with the p2μGALPa-LHP1 plasmid (Figure 3A lane 4), we observe a strong over-accumulation of U3-3′II (and likely of U3-3′I as well) as compared to the CY3 wild-type strain (Figure 3A compare lanes 2 and 4 and see also Supplementary Figure 3A online for quantification of the signals). Since the expression of the Protein A tag alone under the same strong promoter and from the same high-copy vector, has no influence on U3-3′II accumulation (Figure 3A lane 3), we conclude that the over-expression of Lhp1p is likely to be responsible of this effect (see footnotes on Supplementary Figure 3 online). In the presence of the ProtA-At32 protein, the U3-3′II precursor accumulates ~45% of the wild-type levels, which is around three times the residual levels observed in the untransformed CY2 strain (Figure 3A lane 5 and Supplementary Figure 3A online). Expression of At79 gives only a 5% increase in U3′-II levels as compared to the untransformed or Protein A expressing CY2 strain (Figure 3A lanes 1, 3 and 6 and see Supplementary Figure 3A online).

tRNAs are transcribed with 5′- and 3′-extensions and many contain intervening sequences. Removal of the 3′-extension is catalysed by an endonucleolytic event occurring most of the time after 5′-end excision (56,70,71). The La proteins protect the 3′-trailer from exonucleolytic trimming and, at least in yeasts, stimulate its endonucleolytic maturation (15,16,72,73). Although there is no clear defect in mature tRNA levels in the absence of Lhp1p, the mechanism of 3′-end tRNA maturation and the order by which it occurs are both altered. In *lhp1-Δ* background, the mature 3′-end is produced by exonucleolytic trimming, a process leading to characteristic modifications in the accumulation patterns of most tRNA precursors and intermediates [(15,16) and compare also lanes 1 and 2 Figure 3B]. We monitored these patterns for five intron-containing tRNA families upon expression of the plant proteins in the CY2 (*lhp1-Δ*) strain (Figure 3B and Supplementary Figure 3B online). The identities of tRNA precursors were determined based on their relative electrophoretic mobility, hybridization patterns

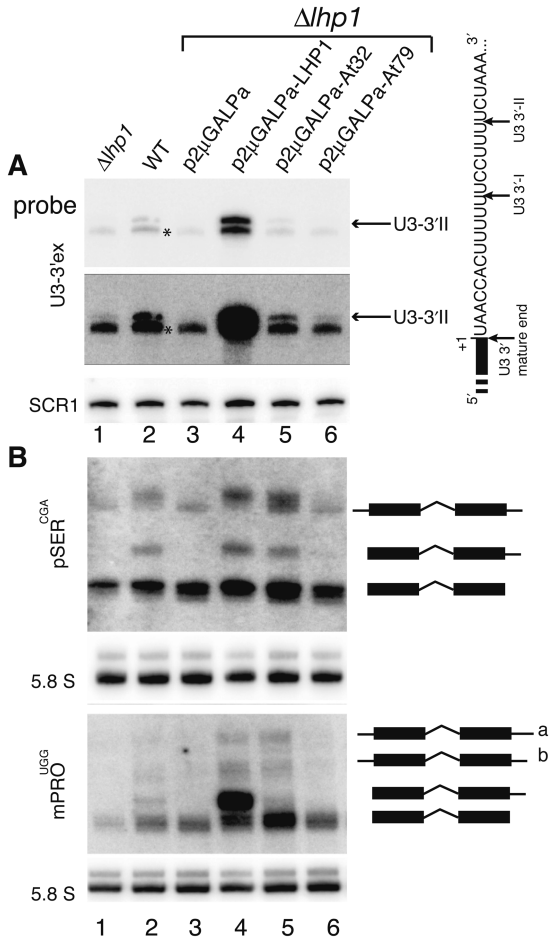


Figure 3. Complementation of *lhp1-Δ* strain pre-U3 and pre-tRNAs processing defects by heterologous expression of At32 and At79. The untransformed *lhp1-Δ* strain (CY2) (lane 1), the isogenic WT strain (CY3) (lane 2), or the CY2 (*lhp1-Δ*) strain transformed with the p2μGALPa set of plasmids allowing the expression of Protein A (lane 3), ProtA-Lhp1p (lane 4), ProtA-At32 (lane 5) or ProtA-At79 (lane 6) were grown in liquid minimal galactose medium and total RNAs extracted for analysis. (**A**) Northern blot analysis of U3 snoRNA precursors pattern. The same blot was probed with an oligonucleotide complementary to the pre-U3 3′-extension (first upper two panels) or to SCR1 (the RNA component of the signal recognition particle) (third panel) as loading control. The second panel corresponds to a longer exposure of the first one. The position of the U3-3′II species is reported on the right and the U3-3′I precursor is marked with an asterisk in the WT lane. The drawing on the right represents a portion of U3 snoRNA 3′-extension with arrows showing the 3′-ends of mature U3 and of U3 3′-I and U3 3′-II precursors, respectively. (**B**) Northern blot analysis of serine^{CGA} and proline^{UGG} tRNAs precursors pattern. Two blots were prepared and respectively probed with oligonucleotides complementary to intronic sequences of ser^{CGA} pre-tRNA (pSER^{CGA}) or to exonic sequences of proline^{UGG} tRNA (mPRO^{UGG}). Each blot was also probed for the 5.8S rRNA as loading control. Schematic representations of tRNA precursors, intermediate and mature species are shown on the right. tRNA^{ProUGG} precursors designated a and b represent two unspliced 5′- and 3′-extensions containing transcripts. Black boxes represent exonic sequences, central broken lines intronic sequences and 5′- and 3′-extensions are depicted by short lines.

and comparison to previous reports (16,56,70). The maturation defects we observed in the *lhp1-Δ* strain for tRNAs serine^{CGA} and proline^{UGG} (Figure 3B lane 1) are as previously reported (16). In these cases, upon Lhp1p depletion, the unspliced 5′- and 3′-extensions containing precursors migrate faster and under accumulate, and the processing intermediates corresponding to unspliced 5′-processed, 3′-unprocessed pre-tRNAs are undetectable (Figure 3B compare lanes 1 and 2).

As for pre-U3 3′-end processing, expression of the Protein A tag alone in CY2 has no impact on the tested pre-tRNA patterns (Figure 3B lane 3 and Supplementary Data Figure 3B online) showing that the effects described below most probably arise from the factor fused to the tag. As expected, expression of the ProtA-Lhp1p in the *lhp1-Δ* background qualitatively restores a wild-type processing pattern for all tested tRNA families (Figure 3B lane 4 and Supplementary Data Figure 3B online). Nevertheless, we can observe a striking over-accumulation of the 3′-extended intermediate of tRNA^{ProUGG} (and of other tested tRNAs, see Supplementary Figure 3B online), a more limited one for tRNA^{SerCGA}, and a slight increase in all unspliced 5′-, 3′-extended transcripts. As for U3, we speculate that all these molecular phenotypes are the consequence of Lhp1p over-expression (see footnotes of Supplementary Figure 3 online).

In the presence of the ProtA-At32 fusion, we clearly observe the restoration of a normal pattern of precursors and intermediates for all tested tRNA families (Figure 3B lane 5 and Supplementary Figure 3B). We also observe, as in the ProtA-Lhp1p-expressing strain but to a more limited extent, the over-accumulation of the 5′-, 3′-extended intron-containing transcripts (Figure 3B compare lanes 2 and 5). Pre-tRNA patterns from the ProtA-At79 expressing CY2 strain are similar to that of the untransformed and Protein A expressing CY2 strains (Figure 3B lane 6 and Supplementary Figure 3B) even after longer exposures (not shown).

Altogether these data show that At32 displays the ability to restore correct precursor patterns of U3 and every tRNA tested. We conclude that At32 is able to restore a wild-type 3′-end maturation process for these precursors and hence to support at least partially Lhp1p function in these processes. On the other hand, expression of At79 in the CY2 background does not allow U3 or tRNA precursors to accumulate. We conclude that expression of At79 is not able to support these Lhp1p functions under such heterologous conditions.

At32 binds to plant RNA polymerase III transcripts *in vivo*

La homologues can associate with highly diverse coding or non-coding RNAs from viral or cellular origins [for review see (5)]. This multi-functionality is in part explained by the capacity of La proteins to specifically bind with high-affinity RNAs presenting a terminal 3′-UUU-OH motif (37,38,40). All primary RNA pol III transcripts end with such a motif but mature forms do not, as it is subsequently removed by a 3′-end processing step. To determine whether At32 is able to bind to 3′-UUU-OH ending RNAs in plant, we asked whether RNA pol III precursors could be co-immunoprecipitated with green fluorescent protein (GFP) tagged versions of the protein. However, since most plant pol III precursors have a very short 3′-extension and are difficult to distinguish

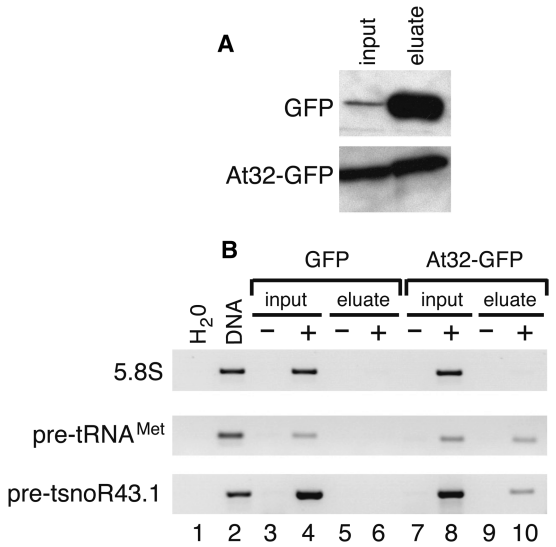


Figure 4. Co-immunoprecipitation experiments with an anti-GFP antibody on crude lysates of GFP or At32-GFP expressing *Arabidopsis* cell lines. (**A**) Western blot analysis of input and immunoprecipitation eluate fractions. Blot was probed with a monoclonal anti-GFP antibody. (**B**) PCR analysis of cDNAs prepared from input and immunoprecipitation eluate fractions. PCR with primer pairs specific to 5.8S cDNA (5.8S), to cDNAs of intron-containing precursors of $tRNA^{Met}$ family (pre-$tRNA^{Met}$) and to the cDNA of the tRNA-snoRNA43.1 precursor (pre-tsnoR43.1) were performed on water or genomic DNA as controls (lanes 1 and 2), on DNase treated but not reverse transcribed input and eluate fractions (minus lanes 3, 5, 7 and 9) and on DNase treated and reverse transcribed input and eluate fractions (plus lanes 4, 6, 8 and 10).

(by hybridization or PCR amplification) from mature forms, we had to select rare situations where this distinction would be possible. One such situation is provided by the presence of an intron in a subgroup of *Arabidopsis* pre-tRNA methionine (pre-$tRNA^{Met}$) (tRNA database: http://lowelab.ucsc.edu/GtRNAdb/) and another by the presence in plant of a dicistronic gene organization, where a tRNA is co-transcribed with a snoRNA in a single precursor molecule (pre-tsnoR43.1) by the RNA pol III complex (74).

Stable transgenic cell suspension lines expressing At32 fused at its NH2 or COOH terminus to the GFP, as well as a cell line expressing the GFP alone as negative control were produced. We prepared native extracts of each of these cell suspension lines and performed immunoprecipitations with anti-GFP antibodies. Two independent experiments that yielded similar results were conducted for each fusion and no significant difference was observed between the NH2 and COOH translational fusions. Western blot analysis performed on identical fractions of each eluate showed that both GFP and At32-GFP are efficiently immunoprecipitated (Figure 4A). We checked for the presence of pre-$tRNA^{Met}$ and pre-tsnoR43.1 in each eluate fraction by RT-PCR analysis (Figure 4B). As negative control, to test the specificity of our immunoprecipitation experiments, we also performed RT-PCR amplifications targeting 5.8S ribosomal RNA since La proteins are not known to associate to this highly abundant transcript. PCR reactions performed on total genomic DNA demonstrate that primers are efficient and give specific signals (Figure 4B lane 2) and since we were not able to amplify from non-reverse transcribed RNAs (Figure 4B lanes 3, 5, 7 and 9), we conclude that the signals obtained with reverse-transcribed samples arise from cDNAs and not from contaminating genomic DNA. Also, in each case, we were able to amplify a specific product using input cDNAs (Figure 4B lanes 4 and 8) showing that each of the three tested RNAs were present in corresponding crude lysates. PCR reactions using cDNAs produced from the At32-GFP immunoprecipitated fraction generated the expected product for pre-$tRNA^{Met}$ and pre-tsnoR43.1, but not for 5.8S rRNA (Figure 4B, lane 10), while PCR reactions using cDNAs produced from the GFP-alone fraction did not produce any PCR product (Figure 4B lane 6). We conclude that pol III transcribed precursors to $tRNA^{Met}$ and tRNA-snoR43.1 specifically co-immunoprecipitate with At32-GFP. Despite the fact that the GFP protein is efficiently immunoprecipitated and is more abundant than At32 in the eluate (Figure 4A), no such RNA is co-immunoprecipitated with GFP alone strongly supporting the conclusion that At32 is able to specifically associate at least to these RNA pol III transcripts *in vivo* and probably to 3′-UUU-OH ending RNAs in general.

To test the capacity of At79 to bind pol III precursor RNAs *in vivo*, we also produced stable transgenic cell suspension lines expressing At79 fused at its NH2 terminus to the GFP protein. Using the same immunoprecipitation procedure as above, we were unable to co-immunoprecipitate pre-$tRNA^{Met}$ or pre-tsnoR43.1 with the At79 fusion protein whereas the protein is efficiently recovered in the eluate fraction (not shown). These negative results were reproduced when performing the PCR reactions on twice the amounts of cDNAs from GFP-At79 eluates.

These results, together with the fact that the At32 is able to complement every tested Lhp1p functions in *S. cerevisiae* strongly suggest that At32 is able to fulfil genuine nuclear La functions in *Arabidopsis* and we hence propose to rename it AtLa1 for *Arabidopsis thaliana* La protein 1. Although displaying characteristic evolutionary and structural features of genuine La proteins, At79 failed to complement yeast phenotypes linked to *LHP1* inactivation and at least in our experimental conditions, does not seem to bind to the tested RNA pol III transcripts in plants.

AtLa1 localizes predominantly to plant nucleoplasm

To get a better understanding of the La function in plant, we further studied AtLa1 in *Arabidopsis*, starting with its subcellular localization. Cell lines expressing GFP (as a control) or AtLa1 fused to the GFP, were prepared as protoplasts and fluorescence was observed by confocal laser scanning microscopy (Figure 5). As expected, the GFP alone is distributed throughout cytoplasm and nucleoplasm with exclusion of the nucleolus (Figure 5A–C). In the vast majority of observed cells, GFP-AtLa1 (NH2 fusion) or AtLa1-GFP (COOH fusion) (Figure 5D–F and not shown) display a diffuse pattern

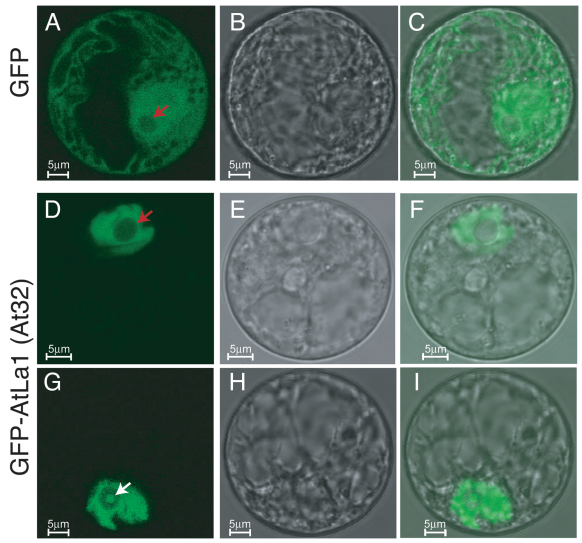


Figure 5. Subcellular distribution of GFP-AtLa1 (At32) fusion protein in *Arabidopsis* cell suspensions. Protoplasts were prepared from *Arabidopsis* cell suspensions stably expressing GFP or GFP-AtLa1 and fluorescent proteins localization assessed by laser confocal microscopy. (**A**–**C**) Protoplast expressing GFP alone. (B) is DIC image of (A), and (C) is merged image of (A) and (B). (**D**–**I**) Protoplasts expressing GFP-AtLa1. (E) is DIC image of (D), and (F) is merged image of (D) and (E). (H) is DIC image of (G), and (I) is merged image of (G) and (H). Red arrows point to the nucleolus and white arrow to the nucleolar cavity.

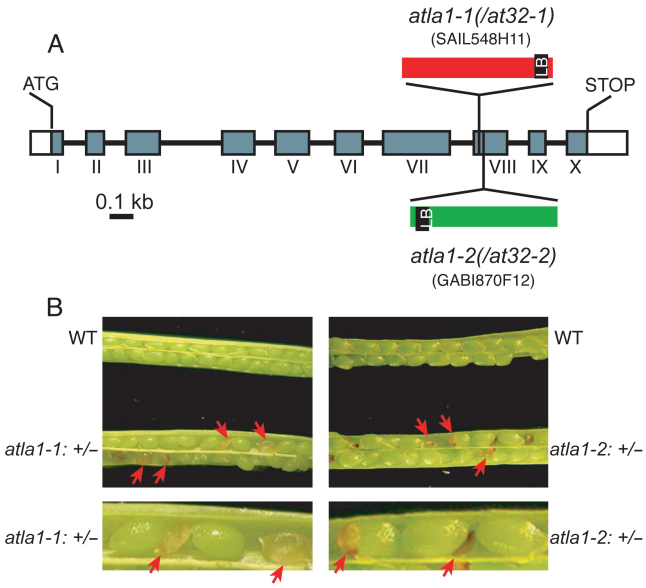


Figure 6. T-DNA insertion in *AtLa1* (/*At32*) gene is lethal. (**A**) Schematic representations of T-DNA insertions at the At4g32720 locus. Comparison of genomic and cDNA (At4g32720.1) sequences revealed that the coding region of the gene contains 10 exons (grey boxes). Open boxes represent 5′- and 3′-untranslated regions. The positions of Sail and Gabi T-DNAs are reported. (**B**) Siliques of hemizygous *AtLa1* insertion lines. Open siliques of self-pollinated *AtLa1*/*atla1-1* (left panels) and *AtLa1*/*atla1-2* (right panels) plants. As a control, siliques of a wild-type plant (*AtLa1*/*AtLa1*) are shown. Red arrows indicate the developmentally arrested siblings.

throughout the nucleoplasm and no obvious general labelling of the nucleolus.

The plant nucleoli have the particularity as compared to other eukaryotes, to display a central entity named nucleolar cavity that appears as a clear space when observed by electron microscopy (75,76). The precise function of the nucleolar cavity is not well defined but amongst other factors small nuclear and nucleolar RNAs were shown to localize in this space (76–78). We observed the accumulation of the AtLa1 NH2 and COOH GFP fusion proteins in the nucleolar cavity for a small fraction of cells (Figure 5G–I and not shown), whereas we never observed the GFP in this nucleolar subcompartment.

This observed subcellular localization is unlikely to be an artefact linked to the production of protoplasts from *Arabidopsis* cells, since the direct observation of a transgenic BY2 tobacco cell line stably expressing GFP-tagged AtLa1 gave the same result (not shown).

AtLa1 is required for viability

To further study AtLa1 functions, we searched several collections of T-DNA insertion mutants for disruption of the At4g32720 locus. We identified two candidates, one from the SAIL (n 548H11) (59) and one from the GABI-KAT (n 870F12) (60) collections. PCR analysis and sequencing allowed us to map each T-DNA insertion sites. Both Sail (allele *atla1-1*) and Gabi (allele *atla1-2*) T-DNAs are inserted at the beginning of the At4g32720 exon VIII, three bases apart (Figure 6A).

Sail and Gabi T-DNAs confer plant resistance to glufosinate (Glufo^r) and sulfadiazine (Sul^r) respectively. Plants bearing *atla1-1* and *atla1-2* alleles can hence be selected for growth on medium containing one of these drugs. After selection for several generations, no homozygous plants were recovered for neither allele, suggesting that the homozygous gene At4g32720 inactivation is lethal. The self-progenies of *AtLa1*/*atla1-1* and *AtLa1*/*atla1-2* always yielded a segregation for drug-sensitive to drug-resistant plants of 1:2 (1135 Glufo^R:601 Glufo^S, $\chi^2 = 1.29$, $P > 0.05$ and 885 Sul^R : 410 Sul^S, $\chi^2 = 1.63$, $P > 0.05$, respectively). These results suggest that the T-DNAs are inserted at a single locus in each case and that the two mutations are nuclear, recessive and sporophytic.

Observation of siliques from hemizygous *atla1* mutant plants under the microscope showed that they contained one-quarter of aborted seeds confirming the lethality of homozygous mutant embryos (Figure 6B and Table 1). Initially aborted seeds appeared smaller and pale brown at a stage where wild-type and hemizygous siblings were green (Figure 6B). Later they were dark brown and completely dried out.

Allelism tests were performed using the two independent T-DNA insertion lines. Immature siliques produced by crosses were opened under the binocular and analysed. The presence of 26.5% of *embryo-defective* seeds (156/589, $\chi^2 = 0.69$, $P > 0.05$) confirmed that the two mutations are allelic and that embryonic lethality directly results from the alteration of AtLa1 function.

Table 1. Comparison of hemizygous siliques from the two mutant lines at the wild-type torpedo/cotyledonary stage

	atla1-1	*atla1-2*
Total number of seeds analysed	533	592
Number of *emb* seeds	137	148
Percentage of *emb* seeds	25.7	25
χ^2(H0 = 1/4) $P > 0.05$	0.14	0
Number of *emb* seeds arrested at early globular stage	83	50
Number of brown and desiccated seeds unusable for phenotype analysis	54	98

atla1 embryos are arrested at early globular stage and display a nucleolar size enlargement

We examined the embryonic phenotype of *atla1* seeds for the two mutant lines (Figure 7 and Table 1). Compared to wild-type embryos at the torpedo/cotyledonary stage, the *atla1* embryos from both lines were much smaller and composed of <50 cells (Figure 7A panels b, c, e and f). Based on their morphology, we conclude that the terminal stage of *atla1* mutant embryo development is early globular (Figure 7A panels c and f).

Comparison of hemizygous siliques from the two mutant lines, taken at the torpedo/cotyledonary stage for wild-type seeds, revealed that the percentage of brown desiccated *atla1-2* seeds is almost twice that of *atla1-1* seeds (Table 1). Moreover embryos proper of *atla1-1* were often marginally larger and composed of more cells than *atla1-2* embryos (Figure 7A compare panels a–c and d–f). These data suggest that although the development of the embryos in both alleles is arrested at the same stage (Figure 7), *atla1-2* mutation is more severe than *atla1-1*.

We also observed that most if not all *atla1* embryo cells display abnormally large nucleoli (Figure 7B). We measured the diameter of nucleoli from *atla1-1* and *atla1-2* embryos arrested at early globular stage (~20 embryos were analysed for each allele with 4–6 nucleoli measured per embryo) and observed that they are 1.8–2 times larger than nucleoli from wild-type embryos at globular stage.

We conclude that the inactivation of AtLa1 function is lethal and leads to an early developmental arrest of the embryo.

DISCUSSION

We report here the identification of two distantly related proteins from *A. thaliana* At32(/AtLa1) and At79 that display every structural feature of *bona fide* La proteins. Microarray expression data from the AtGenexpress project and our western blot analysis clearly indicate that both corresponding loci (At4g32720 and At1g79880) are expressed *in vivo*. Based on its capacity to fulfil in yeast the small nuclear RNA biogenesis function of Lhp1p and to bind *in planta* to RNA pol III transcripts, we conclude that the protein produced at the At4g32720 locus (we named AtLa1 for *Arabidopsis thaliana* La protein 1) is a genuine *A. thaliana* La protein.

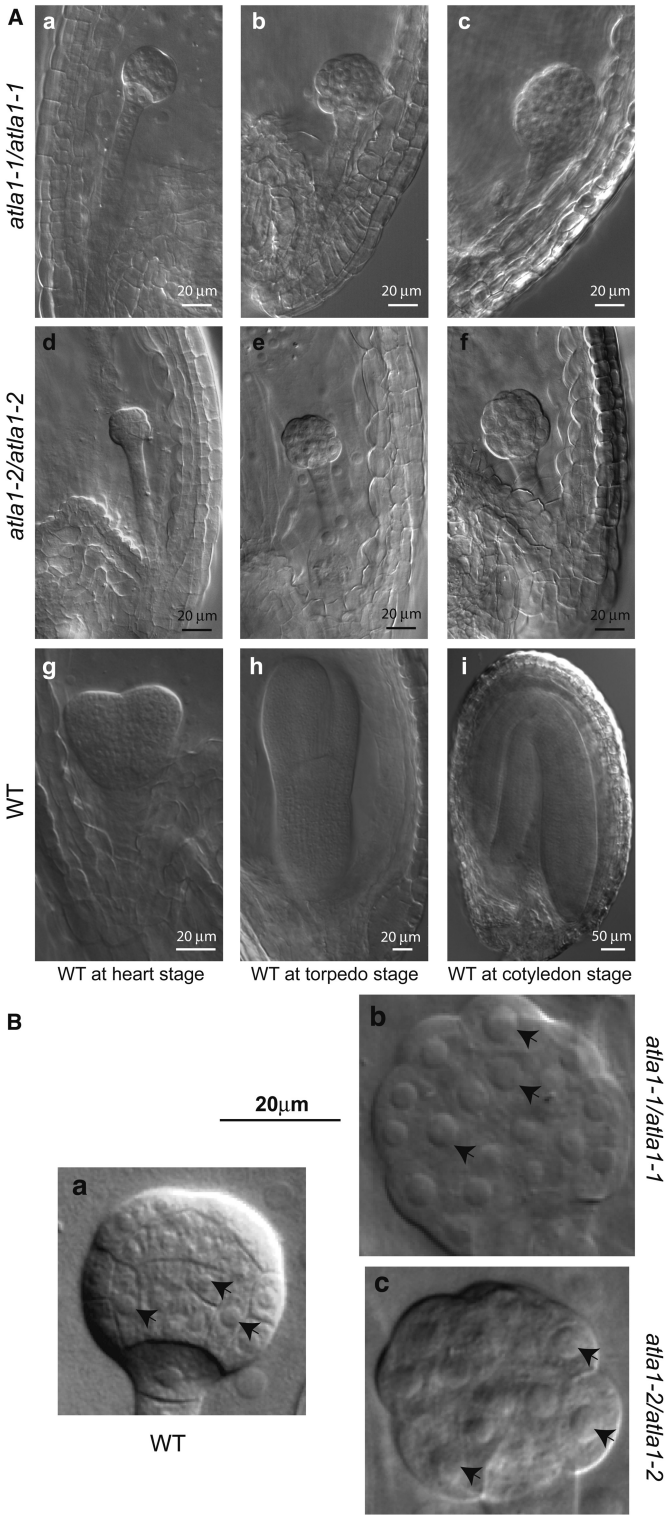


Figure 7. Phenotypes of *atla1* embryos. (**A**) *atla1* mutant embryos are developmentally arrested at early globular stage. Images of cleared seeds containing *atla1-1* embryos (a–c), *atla1-2* embryos (d–f) or wild-type embryos (g–i) are shown. Embryos were analysed at three developmental stages corresponding to wild-type heart-stage (a, d and g), torpedo stage (b, e and h) or cotyledon stage (c, f and i). (**B**) *atla1* mutant cells display a nucleolar size enlargement. Images of cleared seeds containing wild-type (a), *atla1-1* (b) or *atla1-2* (c) embryos at early globular stage. Arrows point to nucleoli. All three images are at the same scale.

The fact that AtLa1 can restore correct U3 and tRNA precursor patterns in *lhp1-Δ* background and growth of the *lsm8-1;lhp1-Δ* strain is a strong indication that it can bind to all RNAs that terminate with an oligouridylate motif (not only to pol III transcripts) and participate in their biogenesis. In yeast, U3 snoRNA is produced by the RNA pol II complex and binding of Lhp1p to precursor molecules is a key step of its maturation process (9). AtLa1 is able to restore correct U3 precursors pattern in an *lhp1-Δ* background showing that it can bind these pol II encoded RNAs and participate in their processing in yeast. This suggests that AtLa1 has the potentiality to bind to *Arabidopsis* pol II-encoded 3′-UUU-OH RNAs such as snoRNAs (79) or snRNAs (80) and to participate in their processing, stability and/or RNP formation.

AtLa1 is also able to restore normal pre-tRNA patterns in an *lhp1-Δ* background and even to stabilize intermediates above wild-type levels. This high level of complementation by a plant protein of the yeast La function in 3′-end pre-tRNAs processing is consistent with previous reports showing that this maturation is evolutionary well conserved in eukaryotes (15,81). This together with the fact that AtLa1 is able to bind *in vivo* to plant tRNA precursors allow us to propose that the mechanism of 3′-processing and protection of pre-tRNAs involves the La function and is conserved in *Arabidopsis*.

Finally, the capacity of AtLa1 to restore growth in the *lsm8-1;lhp1-Δ* strain is a strong indication that it can participate in U6 snRNA biogenesis. In yeast, both the ring-shaped complex Lsm2p-Lsm8p and the Lhp1p proteins are involved in the biogenesis of several non-coding RNAs such as U3 snoRNA, tRNAs and U6 snRNA (18,82,83). However, while *LSM8* or *LHP1* inactivation has no impact on the steady-state accumulation of mature U3 or tRNAs (82,83), U6 snRNA levels are reduced by 50% in the *lsm8-1* background (18,53). A 2–3-fold over-expression of Lhp1p in this background restores U6 snRNA levels to 75% of wild-type and expression of U6 snRNA in extra copies allows viability of the *lsm8-1;lhp1-Δ* strain (18,53). These data suggest that Lhp1p is needed in the *lsm8-1* background to help in U6 snRNP biosynthesis and/or accumulation and the ability of AtLa1 to restore growth of the *lsm8-1;lhp1-Δ* strain is a strong indication that it can at least partially replace Lhp1p in this role.

In summary, our data strongly suggest that in higher plants as in other eukaryotes, oligouridylate ending transcripts, whether encoded by RNA pol II or RNA pol III, require the La function for normal biogenesis and that this function is fulfilled by AtLa1.

A role for AtLa1 in the nucleolar cavity?

Steady-state cellular localization studies using the hLa protein revealed a diffuse nuclear pattern with some labelling of the nucleolus (24,25,84). In fact, hLa was shown to shuttle in and out of the nucleolus (25,26) and its distribution changes throughout the cell cycle with its major pool relocated to the nucleolus during late G1 and early S phases (24). The significance of hLa nucleolar accumulation is not fully understood but it could be involved in early steps of ribosomal RNA biogenesis (84), in the stabilization of some RNA pol III transcripts (such as U6 or pre-tRNAs as part of their nucleolar maturation and/or modification processes) and in binding U3 precursor and forming the corresponding RNP (3,26). As expected for a genuine La protein, we report that AtLa1 mainly localizes to the nucleoplasm in *Arabidopsis* cells. We did not observe a significant level of labelling of the nucleolus using the GFP-tagged AtLa1 proteins under steady-state conditions (except in the nucleolar cavity, see below). In agreement, AtLa1 was not identified in the proteomic analysis of the *Arabidopsis* nucleolus content (85) suggesting that AtLa1 is not a stable component of this nuclear subcompartment. However, it is possible that a small pool of AtLa1 shuttles through nucleolus and/or that the protein accumulates in this compartment at precise steps of the cell cycle. We also observed that, in a small fraction of cells, a significant pool of AtLa1 localizes to the nucleolar cavity. The nucleolar cavity is a highly dynamic entity, devoid of transcription, whose presence and structure is dependent upon cell cycle progression (75,76). Since we did not utilize a synchronous cell population to perform subcellular localization experiments, not every observed protoplast will contain a nucleolar cavity, and since the structure and probably content of this entity varies at least according to the cell cycle, it is possible that AtLa1 does not accumulate in this sub-nucleolar compartment at all times. This explains at least partially why only a sub-fraction of observed cells (whether *Arabidopsis* protoplasts or tobacco cells) displays fluorescent labelling of the nucleolar cavity. Several snoRNAs such as U3, spliceosomal snRNAs or the 7-2/MRP RNA component of the RNase MRP complex were reported to localize at least transiently to the nucleolar cavity (76–78). However, it is neither known if mature or precursor RNAs were detected in these studies, nor if other RNA pol III transcripts such as tRNAs could be present in this structure. The significance and the role of the nucleolar cavity is unknown but it was proposed to be a storage space and/or to host maturation processes (77). We speculate that AtLa1 could fulfil chaperone and/or stabilization functions for RNAs present in this cavity.

AtLa1 function is required early in embryogenesis

The La homologues from budding and fission yeasts are not required for viability in otherwise wild-type conditions (15,69). However, the situation in yeasts, where La proteins have a much shorter C-terminal domain compared to other eukaryotes, is likely to be exceptional. Indeed La homologues from mouse (86), fly (87) and trypanosome (13,88) are required for viability and we show here that this is also the case for AtLa1 in *Arabidopsis*.

The AtLa1 function is apparently not essential during gametogenesis but is required early in embryonic development. *atla1*-deficient embryos cannot reach the globular to heart stage transition and terminate development and desiccate at the early globular stage. This is similar to the situation in mouse where La is required early in development for the production of

healthy blastocysts (86), but contrasts with the *Drosophila melanogaster* situation where La becomes essential only at the late midgut stage (87).

An intriguing characteristic of *atla1* mutant embryos is the presence of unusually large nucleoli. Although a similar nucleolar hypertrophy was previously reported for other *Arabidopsis emb* mutants (*domino1*, *titan* and *plz*) (89–91), it is not a general feature of developmentally arrested embryos. The nucleolus is the site of ribosome biogenesis, and, in wild-type conditions, its size and morphology directly correlates with ribosome production activity (92,93). In genetically altered background, increase in nucleolus size can be a direct consequence of pre-ribosomal RNA processing or ribosome assembly alteration (89,94,95), or can indirectly result in the alteration of other cellular processes interconnected with nucleolar functions (96,97) as this is probably the case in *titan* and *plz* mutants which display, in addition to nucleolar hypertrophy, cell cycle and cytokinesis defects (90,91,98,99). In this case, however, we know that the La protein is likely to be important for ribosome biogenesis. Indeed, the human La protein was reported to interact in the nucleolus with nucleolin (84), a protein involved in ribosomal RNA production, processing, assembly and export of pre-ribosomal particles (100). Also, the yeast Lhp1p protein was shown to participate in the stabilization and folding of 5S rRNA and, when over-expressed, is able to restore 60S ribosomal subunit production in strains expressing *cis*-mutated 5S rRNAs (94). Therefore, we suggest that the nucleolar hypertrophy we observed in *atla1* mutant embryos likely results from a direct rather than indirect effect on ribosome biogenesis and nucleolar functions. However, since AtLa1 is probably a multi-functional protein, the perturbation of nucleolar functions is not necessarily responsible for the lethality phenotype we observed in the mutant.

Two distinct genuine La proteins in *Arabidopsis*?

The presence of two proteins with structural characteristics compatible with the La function is a feature that is likely to be conserved for all higher plants since this situation is present in *Arabidopsis* (a dicotyledon) and rice (a monocotyledon). The two *Arabidopsis* proteins present considerable sequence divergence with a mean amino acid identity of only 44%. This leads us to speculate that this sequence divergence was associated with a certain level of specialization and that the functions of the proteins are at least not fully redundant.

We showed that At79, the second putative *Arabidopsis* La homologue, possesses every structural feature of *bona fide* La proteins. It displays a characteristic La NTD organization and we clearly detected an atypical RRM2 motif in its CTD, which we believe is an additional structural hallmark of genuine La proteins (see Supplementary Figure 1 online). Nevertheless, At79 was not able to complement Lhp1p function in non-coding RNA biosynthesis in yeast, whereas AtLa1 did. This came as a surprise, especially concerning the well-conserved pre-tRNA maturation process (15,81). Several reasons could account why At79 is not functional in yeast among which, improper folding and/or sub-cellular localization of the protein. But we also failed to co-immunoprecipitate 3′-UUU-OH RNAs with GFP-tagged versions of At79 expressed in *Arabidopsis* cell suspensions. Although we cannot strictly rule out the possibility that the presence of the GFP tag has an inhibitory effect on At79's ability to bind to RNA pol III transcripts, we believe it is unlikely since La proteins from different organisms (hLa, *S. pombe* La, Lhp1p, AtLa1) were previously proven functional as GFP fusions [(25,41,101) and our present results]. We therefore conclude from our immunoprecipitation and yeast complementation results that At79 is probably not able to bind to RNA pol III transcripts and is not involved in their biogenesis.

This situation where a protein structurally similar to a genuine La factor is apparently not competent to fulfil the most 'basic' La function, that is to bind to RNA pol III precursor RNAs, is unprecedented and indeed puzzling. However, it is now clear that there is more to the La function than just binding RNA pol III precursors [for review see (5)] and we propose that At79 is not fully redundant with AtLa1 and that this protein specialized to fulfil other aspects of the La function. In support of this hypothesis, the mRNA patterns of expression of both genes are not superimposed across plant development and their levels are not regulated in the same way in different stress situations such as cold and heat shocks (AtGenExpress, data not shown). Moreover, we observed that AtLa1 is essential for viability, whereas At79 is dispensable for normal plant growth and development (not shown), suggesting that At79 is not able to fulfil entirely the AtLa1 function and that there is at least partial specialization of the two La homologues in *Arabidopsis*. This specialization is unprecedented in eukaryotes and studying the La function in plants should provide unique opportunities to dissect the very different aspects of this crucial cellular activity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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