Core spliceosomal Sm proteins as constituents of cytoplasmic mRNPs in plants

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SUMMARY

In recent years, research has increasingly focused on the key role of post-transcriptional regulation of messenger ribonucleoprotein (mRNP) function and turnover. As a result of the complexity and dynamic nature of mRNPs, the full composition of a single mRNP complex remains unrevealed and mRNPs are poorly described in plants. Here we identify canonical Sm proteins as part of the cytoplasmic mRNP complex, indicating their function in the post-transcriptional regulation of gene expression in plants. Sm proteins comprise an evolutionarily ancient family of small RNA-binding proteins involved in pre-mRNA splicing. The latest research indicates that Sm could also impact on mRNA at subsequent stages of its life cycle. In this work we show that in the microsporocyte cytoplasm of Larix decidua, the European larch, Sm proteins accumulate within distinct cytoplasmic bodies, also containing polyadenylated RNA. To date, several types of cytoplasmic bodies involved in the post-transcriptional regulation of gene expression have been described, mainly in animal cells. Their role and molecular composition in plants remain less well established, however. A total of 222 mRNA transcripts have been identified as cytoplasmic partners for Sm proteins. The specific colocalization of these mRNAs with Sm proteins within cytoplasmic bodies has been confirmed via microscopic analysis. The results from this work support the hypothesis, that evolutionarily conserved Sm proteins have been adapted to perform a whole repertoire of functions related to the post-transcriptional regulation of gene expression in Eukaryota. This adaptation presumably enabled them to coordinate the interdependent processes of splicing element assembly, mRNA maturation and processing, and mRNA translation regulation, and its degradation.

Keywords: cytoplasmic bodies, mRNP, cajal bodies, P-bodies, stress granules, RIP-seq.

INTRODUCTION

Post-transcriptional regulation of gene expression plays an essential role in all aspects of the life cycle of a cell, including cell development, differentiation, survival, homeostasis, adaptation to stress and response to environmental signals.

The cytoplasm plays the key role in the regulation of gene expression by controlling the level of mRNA translation and decay. The transport, localization and degradation of mRNA within this compartment are highly ordered processes. This complexity is not surprising considering the high content of mRNA in the cytoplasm (up to 150 000 molecules in mammalian cells; Halbeisen et al., 2008), where two major counteracting processes of mRNA translation and decay must occur at the same time. As stated in the literature, the vast majority of mRNA post-transcriptional regulation occurs within the specialized microdomains referred to as cytoplasmic bodies (Moser and Fritzler, 2010; Lavut and Raveh, 2012). These non-membrane bound, highly dynamic structures enriched in ribonucleoproteins are evolutionarily conserved, as their occurrence has been confirmed in yeasts (Sheth and Parker, 2003; Lavut and Raveh, 2012), protozoans (López-Rosas et al., 2012), nematodes (Barbee et al., 2002; Gallo et al., 2008), insects (Liu and Gall, 2007; Lee et al., 2009),...
amphibians (Bilinski et al., 2004), mammals (Chuma et al., 2003; Sen and Blau, 2005) and plants (Xu and Chua, 2009; Smolířskí et al., 2011; Perea-Resa et al., 2012). Several cytoplasmic bodies have been described to date, mainly in animal cells. These include P-bodies (PBs), stress granules (SGs), neuronal granules, germinal granules and nuage, among others. The role and molecular composition of cytoplasmic bodies in plants remain less understood, however.

P-bodies are sites of mRNA regulation, including 5'-3' deadenylation-dependent degradation (Sheth and Parker, 2003; Beggs, 2005), microRNA (miRNA)-mediated decay (Sen and Blau, 2005), and mRNA stabilization and sequestration (Xu and Chua, 2009; Scarpin et al., 2017), as well as non-sense-mediated decay (NMD; Unterholzer and Iza- uralde, 2004) and translational regulation (Andrei et al., 2005; Kedersha et al., 2005; Wilczynska et al., 2005; Chu and Rana, 2006). As a result of their composition, including proteins involved in both mRNA stabilization and turnover, P-bodies constitute centers of mRNA sorting where transcripts are directed to either translation or degradation (Lavut and Raveh, 2012). It has been revealed that the components of P-bodies accumulate transiently and can shuttle between the cytoplasm and other cytoplasmic granules (Kedersha et al., 2005; Wilczynska et al., 2005; Moser and Fritzler, 2010; Gutierrez-Beltran et al., 2015). Despite relatively broad knowledge on P-bodies in animal cells, their composition and function in plants is much less understood. Many components are shared between animal and plant P-bodies: for example, LSm1-7, DCP1/2, AGO1 and XRN exonuclease (Xu and Chua, 2009; Maldonado-Bonilla, 2014). No homolog of GW182, which is a marker protein for animal P-bodies, has been found in plants, however. Furthermore, plant P-bodies accumulate proteins distinct from their animal counterparts: for example, AtTZF1 and hTTP (Pomeranz et al., 2010; Gutierrez-Beltran et al., 2015). Functionally, plant P-bodies are involved in translational repression and mRNA decapping (Chantarachot and Bailey-Serres, 2018). Mutational studies indicate that plant P-bodies have a developmental rather than housekeeping function, and that their formation is triggered by the active repression of mRNA translation. Null mutants of DCP1 and DCP2 are lethal at the post-embryonic stage in Arabidopsis thaliana, pointing to the role of P-bodies and mRNA decapping in plant early development (Xu et al., 2006). Further studies identified DCP5 as another P-body component in plants that is essential for the translational repression of seed storage protein mRNAs (e.g. OLEO1) and P-body formation in germinating seedlings (Xu and Chua, 2009).

Stress granules (SGs) are evolutionarily conserved cytoplasmic microdomains representing aggregates of translationally stalled mRNPs that are formed upon environmental stress (Weber et al., 2008; Sorenson and Bailey-Serres, 2014). Their formation is triggered by the inhibition of translation initiation of mRNAs (Protter and Parker, 2016). Importantly, upon stress recovery the transcripts stored in SGs might be targeted to PB-mediated degradation or might be released to the cytoplasm for translation (Chantarachot and Bailey-Serres, 2018). The proteomic analysis of isolated SGs from A. thaliana revealed 118 proteins present within these microdomains (Kosmacz et al., 2019). In addition to marker proteins related to SG formation and dynamics (e.g. eukaryotic translation initiation factors elf4A, elf4E and elf3, poly-A binding proteins PAB2, PAB8 and RBP47B, and oligouridi- late-binding proteins UBPIA, UBPIB and UBPIC), a number of enzymes (e.g. those involved in ethylene, glucosinolate and rhamnose metabolism), growth regulators (CDKA1) and other proteins related to plant stress responses (peroxidases, MKK5, MPK3 and SNRK kinases) were identified. It has been demonstrated that SGs are physically, compositionally and functionally linked to P-bodies (Buchan and Parker, 2009; Chantarachot and Bailey-Serres, 2018). A portion of the proteome of SGs overlaps with PBs, such as TUDOR-SN proteins (TSN1 and TSN2) and tandem zinc-finger proteins (AtTZF1, 4, 5, 6 and 9), which localize to SGs upon heat stress (Pomeranz et al., 2010; Gutierrez-Beltran et al., 2015). Thus, the mRNA life cycle involves constant dynamic changes in mRNP composition and subcellular localization, where mRNPs can shuttle between polysomes, P-bodies and stress granules, depending on external stimuli and the current metabolic needs of the cell. Despite growing research on the post-transcriptional regulation of mRNA usage and its spatial organization within the cytoplasm, the full mechanisms and functional roles of mRNP assemblies into higher-order microdomains must be elucidated.

In this study, we show that canonical Sm proteins are part of the cytoplasmic mRNP complex and thus function in the post-transcriptional regulation of gene expression in plants. Sm proteins constitute an evolutionarily ancient family of small RNA-binding proteins. In eukaryotic cells, these molecules are primarily involved in pre-mRNA splicing. Only a few studies have indicated that, in addition to this well-known function, Sm proteins could also impact mRNA at subsequent stages of its life cycle. In our research, using European larch (Larix decidua L.) microsporocytes as a model system, we show that there is a significant pool of Sm proteins that accumulate within distinct cytoplasmic bodies that also contain polyadeny- lated [poly(A)] RNA. A correlation between the cyclic occurrence of the cytoplasmic bodies and the metabolic activity of the cell was shown. The cytoplasmic bodies were localized after transcriptional bursts, indicating that these microdomains are formed as a result of pulsed, extensive mRNA synthesis. The European larch microsporocytes are male germline precursors that have been shown to be
highly active periodically during diplotene (Kolowerzo-Lubnau et al., 2015). The diplotene stage lasts approximately 5 months and it is possible to divide it into several sub-stages and to observe each substage in detail. It has been demonstrated that in *Larix* species, the chromatin is subject to substantial rearrangements and the microsporocyte is highly metabolically active, increasing its volume sixfold during this stage (Zhang et al., 2008; Kolowerzo-Lubnau et al., 2015). In *L. decidua*, metabolic activity is strictly driven by cyclic chromatin contraction and diffusion. Five periods of chromatin relaxation (diffuse stages) separated by four periods of genome contraction are distinguished during diplotene. In many animal and plant species, diplotene is characterized by significant chromatin relaxation, referred to as the diffuse stage of meiosis (Klasterska, 1976; Cenci et al., 1994; Sheehan and Pawlowski, 2009; Comizzoli et al., 2011; She et al., 2013; Colas et al., 2017). As a result, RNA synthesis is not a continuous process but occurs in transcriptional bursts: the diffuse stages are accompanied by large increases in renewed transcription of both mRNAs and rRNAs, as well as the expression and assembly of splicing subunits (Smoliński et al., 2007; Smoliński et al., 2011; Hyjek et al., 2015).

A total of 222 mRNAs were identified as cytoplasmic partners for Sm proteins, and they comprise three major categories of Sm protein-associated mRNAs that code for proteins related to: (i) ribosomes/translation; (ii) mitochondria/energy metabolism; and (iii) chloroplasts/photosynthesis.

Based on the results obtained in this work and studies on other model eukaryotic cells, we propose that Sm protein–mRNA bodies constitute newly described cytoplasmic domains involved in the post-transcriptional regulation of highly expressed transcripts, particularly in cells in which mRNA synthesis occurs in transcriptional bursts.

**RESULTS**

**S-bodies: cytoplasmic bodies rich in Sm proteins and polyadenylated mRNA**

The specificity of Y12 antibodies against larch Sm proteins has been determined previously (Smoliński et al., 2011). In order to validate the specificity of the anti-Sm antibodies used in these studies, immunoprecipitation and Western blot analyses were performed using cytoplasmic protein extract from larch anthers. All of the immunoglobulins tested specifically precipitated the SmB protein, with the Y12 antibody (not commercial) also effectively precipitating the low-molecular SmD1–SmD3 proteins (Figure S1). In order to further confirm the specificity of the antibodies, the identification of proteins after immunoprecipitation (IP) was performed by mass spectrometry (using the IP-SDS-PAGE-MS method). On the basis of the results obtained, in conjunction with the predicted mass of the studied polypeptides, seven of the eight core Sm proteins were identified: SmF (11 kDa), SmE (12 kDa), SmD1 (13 kDa), SmD2 (14 kDa), SmD3 (16 kDa), SmB (25 kDa) and SmB’ (26 kDa) (Figure S2).

A double labeling assay of the core spliceosomal Sm proteins and poly(A) RNA revealed a remarkable distribution of these molecules within the cytoplasm of microsporocytes (Figures 1 and S3). In the larch anther, cytoplasmic bodies containing Sm proteins were limited to the microsporocyte cytoplasm. Other anther cells (epidermal cells, middle layer or tapetum cells) were devoid of Sm protein concentration in the cytoplasm (Figure S3).

Distinct 0.5–1.0 μm cytoplasmic foci were observed, in which a significant quantity of polyadenylated transcripts had accumulated, in colocalization with Sm proteins. To verify the specificity of the observed labeling, three different antibodies were used for Sm protein localization, namely, commercially available Y12 MAbS (Figure 1a), Y12 MAbs from Matera’s laboratory (Figure 1b) and the ANA No. 5 human reference anti-serum (Figure 1c). All of the antibodies used showed a similar pattern of labeling, with a well-established and characteristic nuclear pool of Sm proteins present in the nucleoplasm and Cajal bodies (CBs), which are highly conserved nuclear domains involved in RNA metabolism, and with the maturation of spliceosomal units after being reimported from the cytoplasm (Darzaq et al., 2002; Staněk and Neugebauer, 2006; Bassett, 2012). Moreover, numerous discrete foci of Sm protein accumulation were observed in the cytoplasm and colocalized with poly(A) RNA (Figure S3). The ultrastructural analysis revealed spherical, non-membrane-bound microdomains with a diameter of 0.4–1.0 μm (Figure 1d,e). The immunogold assay confirmed that a noticeable portion of the Sm proteins accumulated within distinct cytoplasmic structures (Figure 1f,g).

The next aim was to examine whether the observed cytoplasmic accumulations are functionally related to other microdomains, such as P-bodies or stress granules. The cellular localizations of well-established markers for P-bodies (DCP1 and DCP5) (Xu and Chua, 2009; Jang et al., 2019) and stress granules (PAB2) (Dufresne et al., 2008; Merret et al., 2013) were investigated. All of the antigens tested were specifically localized to the cytoplasm (Figure 2). In periods when the Sm-rich cytoplasmic bodies were visible, DCP1 was distributed in a disperse manner throughout the cytoplasm and no distinct accumulations of P-body markers could be observed (Figure 2a). Remarkably, P-bodies were easily detected during periods of nuclear localization of the Sm proteins. Numerous cytoplasmic accumulations of DCP5 were observed at this stage, albeit showing no colocalization with Sm or poly(A) RNA (Figure 2b). Interestingly, the distribution of the polyadenylated RNA and the decapping machinery was clearly spatially separated. Although the cytoplasmic pool of poly(A) RNA was...
dispersed around the nucleus, the P-bodies were located at the periphery of the cytoplasm (Figure 2b). For PAB2, only diffused fluorescence was visible in the cytoplasm (Figure 2c). The lack of distinct accumulations of PAB2 demonstrates that the microsporocytes do not form SGs during diplotene. This was expected, as the cells were not subject to stress conditions prior to analysis. Despite no significant accumulation of PAB2 within Sm-rich cytoplasmic bodies, sometimes the colocalization of this protein with Sm accumulations could be observed, although the concentration of PAB2 was similar to that in the surrounding cytoplasm (Figure 2d). We attribute this to some residual pool of the poly(A) RNA that is still bound to PAB2 within the cytoplasmic bodies.

In summary, none of the protein markers investigated accumulated within the observed cytoplasmic bodies, indicating that they are not compositionally related to PBs or SGs. This conclusion is further confirmed by the fact that DCP1, DCP5 and PAB2 did not coprecipitate with Sm proteins from larch anthers (Table S2). Based on these observations and taking into consideration the general nomenclature used for this type of microdomain, we propose the name of S-bodies for the structures observed in larch microsporocyte cytoplasm.

**S-bodies are formed during the cytoplasmic export of poly(A) RNA**

Larch microsporocytes exhibit a rigorously regulated pattern of metabolic activity. During the diplotene stage of meiosis, five bursts of de novo synthesis of poly(A) RNA occur, separated by periods of transcriptional silencing (Kolowerzo-Lubnau et al., 2015). Each of these cellular poly(A) RNA turnover cycles comprises subsequent stages of nuclear synthesis, cytoplasmic export and degradation of mRNA (Figure 3). A detailed microscopic analysis showed that the S-bodies emerge periodically in each of the five cycles of poly(A) RNA turnover. Based on the transcription rate and localization of the poly(A) transcripts, the longest and most intensive fourth cycle could be divided into seven stages. Shortly after intensive synthesis in the nucleus (stages I and II; Figure 3a), poly(A) RNAs are transported to the cytoplasm, as indicated by an increasing level of labeling in this compartment (stages III–VII; Figure 3a). A remarkable ring-like accumulation of poly(A) RNA was observed around the nucleus, reflecting the pool of newly exported transcripts. Additionally, starting at stage III of the cycle, distinct foci of the poly(A) RNA and Sm proteins were observed and represented the S-bodies. At stage IV, the number of bodies was the highest observed in the whole cycle (380 ± 35 bodies per cell; Figure 3b). During the subsequent stages of cytoplasmic RNA localization, this number consequently decreased (stages V–VII; Figure 3b). It should be emphasized, however, that throughout the whole period when S-bodies were observed, there was a significant colocalization of both poly(A) RNA and Sm proteins (Pearson correlation coefficient, PC = 0.69 ± 0.02; Figure 3d) in the cytoplasmic pool. Furthermore, the Manders’ overlap calculation demonstrated that a substantial fraction of Sm proteins in the cytoplasmic pool had colocalized with poly(A) RNA (67.43 ± 2.90%; Figure 3e).
The overlap coefficient gradually increased as the cytoplasmic export of mRNAs proceeded: from 35.76 ± 3.09% at stage III to 88.03 ± 2.13% of the total cytoplasmic Sm protein pool at stage VI (Figure 3e). We also estimated the percentage of the cytoplasmic poly(A) RNA pool present in S-bodies during the fourth cycle of transcriptional activity at the diploidene stage. The analysis revealed that 3.08 ± 0.42% of the total cytoplasmic pool of poly(A) RNA had accumulated in cytoplasmic bodies, and this accumulation was consistent throughout the cytoplasmic phase of the RNA cell cycle (differences between stages were not statistically significant: analysis of variance, ANOVA, \( P > 0.05 \); Figure 3f).

To test the relationship between the occurrence of cytoplasmic bodies and the metabolic state of the cell, a quantitative analysis of the marker for transcriptional activity (active RNA pol II) was performed in the three cycles with the most intensive poly(A) RNA turnover (Figure 4). During the stages of RNA cytoplasmic export (stages III–VII; see Figure 3a), that is, when the Sm proteins and polyadenylated transcripts accumulate within cytoplasmic bodies, the transcriptional activity was considerably decreased compared with the transcriptional activity in the nuclear stages of mRNA synthesis (stages I and II; see Figures 3a and 4a). In contrast, the level of the marker for potential translational activity (5S rRNA) showed no correlation (Figure 4b). These results indicate that the formation of S-bodies in the cytoplasm is slightly postponed with respect to bursts of nuclear mRNA synthesis, and that
Figure 3. The spatial and temporal distribution of Sm proteins in the fourth cycle of poly(A) RNA turnover. (a) Localization of Sm proteins in the fourth cycle of poly(A) RNA turnover. I–VIII: successive stages of the cycle of poly(A) RNA turnover. A detailed description is provided in the text; CB, Cajal body. Scale bars: 15 µm. (b) The mean number of S-bodies per cell in subsequent stages of the cycle of poly(A) RNA turnover. Error bars indicate standard errors of the mean (SEMs). P < 0.05. (c) The mean volume of S-bodies in subsequent stages of the cycle of poly(A) RNA turnover. (d, e) Analysis of the colocalization of poly(A) RNA and Sm proteins in the cytoplasm at subsequent stages of the poly(A) RNA cycle in the cell. The values are shown as means ± standard errors of the mean (SEMs); PC, Pearson colocalization coefficient. (f) The percentages were calculated on the basis of the Manders’ overlap coefficient, in which the value of 1 was converted to 100% colocalization. Error bars indicate the standard errors of the mean (SEMs); a–b, P < 0.01; a–c and b–c, P < 0.001.
these microdomains appear in periods of decreased transcriptional activity.

**S-bodies are not sites of spliceosomal particle accumulation**

The well-known and broadly documented role of Sm proteins in the nucleus is their participation in pre-mRNA splicing. Sm proteins bind uridine-rich small nuclear RNAs (U snRNAs) in the form of a heptameric ring around the transcript, creating the core spliceosomal ribonucleoparticle (U snRNAs) in the form of a heptameric ring around the transcript, creating the core spliceosomal ribonucleoparticles U1, U2, U4 and U5 snRNPs (Zieve et al., 1988; Baserga and Steitz, 1993; Raker et al., 1999; Shaw et al., 2008). The assembly of U snRNPs involves a highly dynamic cytoplasmic stage at which the Sm proteins are loaded onto the U snRNA transcripts and the 5’ trimethyl-guanosine (m3G) cap is formed (Mattaj, 1986; Urlaub et al., 2001; Yong et al., 2010). Moreover, our previous research (Hyjek et al., 2015) revealed that, during larch meiosis, the cytoplasmic stage of U snRNP assembly can occur in two spatially distinct manners: it may be diffused throughout the cytoplasm or localized within snRNP-rich cytoplasmic bodies (CsBs). The assembly mode depends on the rate of de novo U snRNP formation and the level of U snRNP in the nucleus. Thus, it could not be precluded that the S-bodies are related to the assembly and/or sequestration of spliceosomal elements. To test this possibility, the cellular distribution of both the nucleotide (U1, U2, U4 and U5 snRNA and m3G cap) and protein (U2B*) components of the U snRNPs were analyzed in relation to the S-bodies (Figures S5 and S4). All the spliceosomal components were specifically localized within the nucleus, with significant accumulation in the CBs. In contrast, no signal in the form of distinct clusters of U snRNA, m3G cap or U2B* protein was observed in the cytoplasm, indicating that the formation of S-bodies does not coincide with the stage of U snRNP assembly in the cytoplasm. The lack of any U snRNP components and the presence Sm proteins in the S-bodies demonstrates that the bodies represent the cytoplasmic pool of canonical Sm proteins acting outside of the spliceosome, and that this localization is presumably related to mRNA.

**Identification of cytoplasmic mRNA transcripts associated with Sm proteins**

To identify the mRNA transcripts that accumulate within S-bodies, we employed an RNA immunoprecipitation (RIP) approach against the Sm proteins from the larch microsporocyte cytoplasm, followed by high-throughput sequencing of the immunopurified RNAs (RIP-seq).

As all three anti-Sm antibodies used for the assays in situ could label the cytoplasmic bodies with comparable specificity and strength (see Figure 1), an additional validation of the results from the RNA immunoprecipitation analysis was performed to select the most efficient antibody to use for further analysis (Figure 6a). The enrichment of U2 snRNA, a canonical cellular partner of the Sm proteins, was estimated and the results were normalized to 5S rRNA (which does not interact with Sm proteins). The experiment revealed that the Y12 MAbs obtained from Matera’s laboratory was the most effective for the RIP assay, precipitating over 1130 times more U2 snRNA than the control (mock IP, no antibody added). This was significantly different than the B4- and 12-fold enrichment obtained using ANA No. 5 and the commercially available Y12, respectively.

The Sm protein-associated transcripts are listed in Appendix S1. A total of 245 transcripts were identified as cytoplasmic partners of Sm proteins, 222 of which were classified as mRNAs. Most of the transcripts were homologous to *Pinaceae*. The gene ontology (GO) functional annotation revealed that the cytoplasmic Sm interactome...
consisted of mRNAs encoding a vast range of proteins, localizing to many cell compartments and linking a number of metabolic processes. A total of 141 sequences (58%) were annotated with 728 GO terms. This functional analysis revealed three major categories of Sm protein-associated mRNAs, and they coded for proteins related to: (i) ribosomes/translation; (ii) mitochondria/energy metabolism; and (iii) chloroplasts/photosynthesis (Figure 6b–d).

To test whether the immunoprecipitated mRNAs are indeed accumulated within S-bodies, the spatial distribution of the transcripts was analyzed in situ. From the 222 Sm protein-associated mRNAs, 16 were selected representing different cellular functions and localizations. A double labeling assay of the larch microsporocytes was performed for the Sm proteins and each of the selected mRNAs (Figures 7 and S5).

The microscopic analysis confirmed the specific localizations of the 13 mRNAs within the cytoplasmic bodies. This specific distribution pattern was observed for mRNAs that encoded diverse proteins, including those involved in translation (Figures 7a and S5a,c,d), energy metabolism (Figures 7b and S5f) and photosynthesis (Figures 7c and S5i), as well as structural proteins of the cytoskeleton (Figures 7d and S5b). Additionally, S-bodies were enriched in

Figure 5. S-bodies are not sites of the U snRNP assembly. Labeling of Sm proteins in colocalization with selected splicing elements. There was no accumulation of U1 snRNA (a), m3G cap (b) or U2B″ proteins (c) in the S-bodies (arrowheads). The right-hand panel represents the magnification of the fragments of the cytoplasm, which are marked with a square; CB, Cajal body; T, anther tapetum cell. Scale bars: 10 μm.
several mRNAs related to protein folding (Figure S5g,h), proteasomal degradation (Figure S5j) and miRNA biogenesis (Figure S5e).

A different pattern of localization was observed for only three mRNAs (Figure S5k–m). Transcripts encoding cellulase (Figure S5k) and tubulin (Figure S5l) showed a diffuse pattern of cytoplasmic distribution, with no distinct concentration in any particular area. Double labeling of these mRNAs with Sm proteins revealed that both of them were found in the cytoplasmic bodies, although without noticeable accumulation (i.e. there were no differences between the level of staining within the cytoplasmic bodies and in the surrounding cytoplasm). For the mRNA that encodes a light-independent protochlorophyllide oxidoreductase (LI-POR), the level of staining was below the limit of detection (Figure S5m).

In the next step, we performed an additional analysis of the SmD mRNAs that were identified in the cytoplasmic transcriptome of the larch microsporocytes, but not enriched in the Sm protein-immunoprecipitated pool of transcripts obtained via RIP-seq assay. The lack of cytoplasmic accumulation within these microdomains was observed in the case of mRNAs encoding SmD1, SmD2 and SmE proteins (Figure S6). This finding demonstrates that the S-bodies are not sites of de novo Sm protein synthesis.

The cytoplasmic Sm proteins interactome
In order to explore the Sm proteic interactome, the cytoplasmic fraction of the larch anther cells was subjected to anti-Sm immunoprecipitation, which was subsequently assessed by mass spectrometry. A total of 118 proteins were detected, of which five represented canonical Sm proteins (SmD1a, SmD1b, SmD3, SmD3b and SmB; Tables S2 and S3). Interestingly, there was a trend similar to the results obtained for mRNA identified by RIP-seq. Similar to the Sm protein RNA interactome, a significant fraction of the proteic interactome included proteins related to: (i) ribosomes/translation (eIF2/IF5, RL10e, RS1, RS3, RS28 and OVA2); (ii) mitochondria/energy metabolism (SHM1 and SHM2 methyltransferases, IDH dehydrogenases and ATM1 ABC transporters); and (iii) plastids/photosynthesis (RCA RuBisCO activase and TKL1 transketolase) (Tables S2 and S3). Furthermore, two RNA-binding protein families were identified as coprecipitating with Sm proteins: Alba (acetylation lowers binding affinity) and ECT (evolutionarily conserved C-terminal region). Alba refers to a broad group of RNA- and DNA-binding proteins implicated in chromatin organization, the regulation of transcription and translation, and RNA metabolism (Goyal et al., 2016). It has been shown that these proteins are involved in the regulation of plant cell development, where
Figure 7. In situ localization of mRNA precipitating with Sm proteins. The panels represent stages IV–VI of poly(A) RNA turnover. Numerous clusters of mRNA that colocalized with the Sm proteins (arrowheads) are visible in the cytoplasm. The right-hand panel represents the magnification of the fragments of the cytoplasm, which are marked with a square. (a) RL 37a, large ribosome subunit protein 37a; (b) NAD7, NADH 7 dehydrogenase subunit; (c) LHCP, protein binding chlorophyll a–b; (d) KIN12b, kinesin 12b-like protein. CB, Cajal body. Scale bars: 10 μm.
they perform essential roles in sperm cell specification in A. thaliana (Borg et al., 2011). Fewer ECT proteins have been investigated compared with mRNA-binding proteins. The scarce ECT research indicates the role of ECT1 and ECT2 in calcium signal transduction from the cytoplasm to the nucleus that affects gene expression (Ok et al., 2005).

**DISCUSSION**

**S-bodies: novel cytoplasmic mRNA domains in plant cells**

Thus far, no distinct Sm proteins and mRNA accumulation has been observed in the cytoplasm of plant cells; however, a growing body of evidence shows a specific localization of Sm proteins and particular mRNAs in animal germ granules, which has implications for transcript localization, translational control and mRNA turnover. It was demonstrated that SmE and SmG, localizing to P-granules in Caenorhabditis elegans, are involved in transcriptional quiescence in germline precursors, and that this coordination of germline differentiation is splicing independent (Barbee et al., 2002; Barbee and Evans, 2006). P-granules are structurally and functionally related to nuages in Xenopus oocytes, where the Sm proteins, but not the other splicing elements, are localized. It was proposed that Sm proteins facilitate the transport of the mRNAs specific to germ granules (e.g. Xcat2) from the nucleus to the cytoplasm (Bilinski et al., 2004). In mouse spermatocytes, Sm proteins accumulate in the RNP-rich chromatoid body (Biggiogera et al., 1990; Moussa et al., 1994) and form a complex with a marker protein of these domains, MTR1 (Chuma et al., 2003). In Drosophila melanogaster, the accumulation of SmB and SmD3 at the posterior of the developing oocyte is directly linked to the unique localization and polarization of oskar mRNA. The proper localization of this transcript is crucial for germ-cell specification during early embryogenesis (Gonsalvez et al., 2010; Jaglarz et al., 2011). Germline and early embryonic cells are highly metabolically active, with a spatiotemporally ordered pattern of expression for distinct groups of developmental genes. The European larch microsporocytes are male germline precursors that have been shown to be highly active periodically during diplo-tene (Koloverzo-Lubnau et al., 2015). In this context, the S-bodies are similar to the germ granules observed in animals and might be considered, to some extent, as their plant counterparts.

**Sm proteins as a part of the cytoplasmic mRNP complex**

A growing body of evidence suggests additional roles for the canonical Sm proteins outside of the spliceosome in the processing, localization and translational control of mRNPs. Little is known about which mRNPs in the pool of all transcripts are regulated by Sm proteins, however, and what is the mechanism of their interaction. To determine which mRNAs are accumulated within the S-bodies, we performed a transcriptomic analysis of cytoplasmic Sm protein-mRNP complexes, followed by in situ detection analysis. The functional annotation showed that three major classes of Sm protein-associated mRNAs could be distinguished, namely, those encoding proteins related to: (i) ribosomes/translation; (ii) mitochondria/energy metabolism; and (iii) chloroplast/photosynthesis. This finding is in agreement with the results obtained for Drosophila ovaries and HeLa cells. Lu et al. (2014) demonstrated that among 72 and 30 Sm protein-associated, fully spliced, polyadenylated mRNAs for Drosophila and HeLa cells, respectively, a significant portion encoded ribosomal and mitochondrial proteins. Some of the mRNAs show overlap with all of the Sm protein interactomes studied to date – plant (this work), insect and mammalian (Lu et al., 2014) – including those that encode small and large ribosomal subunit proteins, translation initiation factor eIF2β, NADH dehydrogenase and cytochrome oxidases. Furthermore, Sm proteins were also associated with the mRNA products of intronless genes (e.g. histone H2A mRNAs in human cells and 60S ribosomal L37 mRNA in larch cells), proving that this interaction is completely independent of pre-mRNA splicing. The results from the animal cells do not clarify whether the Sm protein-associated mRNAs are localized to the nucleus or to the cytoplasm, as the RIP-seq was performed on whole-cell extracts. Nevertheless, together with the aforementioned reports, our results indicate that the association between splicing-independent Sm proteins and mRNA is conserved in invertebrates, vertebrates and plants.

Mutual post-transcriptional coordination of mRNA sub-populations has been previously described by Keene and Tenenbaum (2002). The following hypothesis was proposed based on eukaryotic post-transcriptional regulons (or operons): structurally and functionally related genes are regulated in groups by specific RNA-binding proteins to facilitate the organization of gene expression during cell growth and development (Keene and Tenenbaum, 2002; Keene, 2007). The most acknowledged RBPs involved in this type of regulation are Pumilio proteins (PUF) that participate in the post-transcriptional silencing of particular groups of genes in yeasts (Gerber et al., 2004), Drosophila (Gerber et al., 2006), mammals (Bohn et al., 2018; Goldstrohm et al., 2018) and plants (Tam et al., 2010). The post-transcriptional regulons also operate in a number of developmental processes and stress responses (Gorospe, 2003; Mazan-Mamczarz et al., 2003; Lü et al., 2006), including the immunological response in mammals (Lai et al., 1999; Ule et al., 2003; Cheadle et al., 2005; Lykke-Andersen and Wagner, 2005). In HeLa cells, the spliceosomal proteins U2AF (U2 auxiliary factor) and PTB (polypyrimidine tract binding protein) bind distinct groups of mature mRNAs in both the nucleus and the cytoplasm (Gama-Carvalho et al., 2006). Whereas U2AF interacts predominantly with...
transcriptional factors and cell cycle regulator mRNAs, PTB-bound transcripts encode proteins related to intracellular and extracellular trafficking and apoptosis. Moreover, Ser-Arg-rich (SR) spliceosomal proteins are linked to the cytoplasmic export of intronless histone mRNAs (Huang and Steitz, 2001; Ankö et al., 2012), the stimulation of translation (Sanford et al., 2004) and the cytoplasmic degradation of particular transcripts (Lemaire et al., 2002). Recently, the involvement of SmD1 in post-transcriptional gene silencing (PTGS) was revealed in *A. thaliana* (Elvira-Matelot et al., 2016). Based on the results from a genetic screening of PTGS-defective mutants, it was proposed that, in addition to its role in pre-mRNA splicing, SmD1 facilities cytoplasmic PTGS. By protecting transgene-derived aberrant RNAs from degradation by the RNA quality control pathway (RQC) in the nucleus, SmD1 presumably promotes cytoplasmic export of transcripts to siRNA bodies, where PTGS occurs (Elvira-Matelot et al., 2016). Thus, the involvement of the evolutionarily conserved canonical Sm proteins in the splicing-independent post-transcriptional control of mRNA metabolism is emerging as another regulatory component of gene expression. Taken together, these findings suggest that Sm proteins may represent a novel example of RNA-binding proteins that regulate functionally related groups of mRNAs within the cytoplasm. In metabolically active microsporocytes (which enlarge sixfold during diplotene; Kołovero-Lubna et al., 2015), the most abundant groups of transcripts are those related to efficient translation, energy production and photosynthesis, which aligns with the results presented.

Therefore, it might be assumed that the Sm protein–mRNA interaction within a cell is a ubiquitous process, applying to a vast number of transcripts.

**The mechanism of Sm protein–mRNA interaction**

An open question concerns the mechanism governing the selectivity of Sm proteins towards certain types of mRNAs and the mechanisms for their interactions. The only Sm protein-binding elements described to date are the Sm sites present in U snRNAs and yeast telomerase RNA (TLC1 and TER1) (Seto et al., 1999; Tang et al., 2012), and in the CAB box found in H/ACA CB-specific RNAs (U85, U87 and U89 small cajal body-specific RNAs [scaRNAs]) (Darzacq et al., 2002; Richard et al., 2003) and human telomerase RNA (hTR) (Fu and Collins, 2006). Sm sites or CAB box domains have not been found in mRNA to date. Furthermore, the specificity of scaRNA binding by Sm proteins is not fully understood. In both *Drosophila* and HeLa cells, Sm proteins coprecipitated only a fraction of the scaRNAs, with no apparent structural features that could explain this selectivity (Lu et al., 2014). Several studies have demonstrated the participation of spliceosomal U1 snRNPs in splicing-independent post-transcriptional regulation of gene expression, including nascent mRNA protection from premature cleavage and polyadenylation at cryptic sites (Kaida et al., 2010; Berg et al., 2012), regulation of polyadenylation of retroviral RNA (Ashe et al., 1997; Ashe et al., 2000; Schrom et al., 2013) as well as control of RNA pol II promoter directionality and promotion of sense mRNA transcription (Almada et al., 2013; Nitini et al., 2013). Recently, it was revealed that in plants U1 snRNPs regulates miRNA biogenesis (Knop et al., 2017; Bhat et al., 2019) and mediates the interactions between the microprocessor, spliceosome and polyadenylation machinery (Szwejkowska-Kulinska et al., 2013; Stepien et al., 2016). Moreover, it was shown that in animal cells the interaction between Sm proteins and mRNAs is mediated by U1 and presumably other snRNPs. Twelve-nucleotide putative binding sites for the 5' end of the U1 snRNPs were identified in Sm protein-associated mature mRNAs within the coding sequence, far from intron-exon boundaries. It was thus proposed that U1 snRNPs bind mature mRNAs to promote the recruitment of RNA processing factors, thereby affecting mRNA localization, translation and/or turnover (Lu et al., 2014). In the case of the larch microsporocytes, the Sm protein–mRNA interaction is apparently not mediated by U snRNPs, as there was no enrichment in U1 snRNA in the RIP-seq analysis. Additionally, the *in situ* localization of splicing elements demonstrated that the U snRNAs and m3G caps did not colocalize within the S-bodies, suggesting a different mechanism of Sm protein–mRNA interaction in these cells. Studies on *Drosophila* and *C. elegans* have revealed that only some Sm proteins associate with mature mRNAs (Barbee et al., 2002; Gonsalvez et al., 2010; Gonsalvez and Long, 2012). Sm proteins constitute an evolutionarily conserved family and are classified into canonical (spliceosomal) Sm and noncanonical LSm (Sm-like) proteins (Hermann et al., 1995; Urlaub et al., 2001; Khusial et al., 2005; Scofield and Lynch, 2008). In Archaea, LSm proteins participate in tRNA maturation and ribosome synthesis (Mura et al., 2003). In Eubacteria, the Hfq homolog of the Sm protein regulates mRNA translation (Schumacher et al., 2002). The diversification of the ancestral Sm proteins in eukaryotes enabled their involvement in various processes. The LSm 2-8 ring is found in U6 and U6 atac snRNPs (spliceosomal subunits), as well as in the U8 small nucleolar ribonucleoproteins (snoRNPs) involved in 5.8S and 28S rRNA processing (Peculis, 1997; Achsel et al., 1999). LSm 1-7 forms a cytoplasmic complex that promotes mRNA decapping in P-bodies (Bouveret et al., 2000; Decker and Parker, 2012). Another LSm ring variant, LSm 2-7, binds snR5 snoRNA, which is essential for rRNA pseudouridylation (Fernandez et al., 2004). The unique LSm 10 and LSm 11 proteins are part of the U7 snRNP involved in the 3' end maturation of histone mRNA in animal cells (Schumperli and Pillai, 2004). The Arabidopsis genome contains 42 Sm and LSm protein genes (Cao et al., 2011), many of which have not yet been
characterized. Taking these findings into consideration, it could not be precluded that in larch microsporocytes the Sm protein–mRNA association is driven by another non-canonical Sm/LSm ring or is indirectly mediated by other RBPs/RNPs. This hypothesis is partially supported by an IP-MS analysis that revealed a significant enrichment of the Alba proteins within the cytoplasmic Sm interactome. Alba proteins constitute an evolutionarily ancient family of dimeric nucleic acid-binding proteins present in all domains of life (Goyal et al., 2016). With their broad functional plasticity, Alba proteins are implicated in genome organization, transcription regulation, RNA metabolism, cell differentiation and stress responses. Structurally, plant Alba paralogs display extensive diversity. Their association with target RNA is sequence independent, and the formation of the Alba–RNA complex facilitates the accessibility of the transcript for RNA processing factors (Goyal et al., 2016). It has been shown that the Alba interactome comprises a vast range of different RNAs and RBPs, including mRNA regulators such as poly(A)-binding proteins (PABPs), ribosomal subunits and translational factors (Guo et al., 2003; Subota et al., 2011; Gissot et al., 2013). Furthermore, the formation of a particular complex within a cell depends on Alba protein abundance and local concentration, the composition of the dimer (homo or hetero) and post-translational modifications, including arginine methylation (Lott et al., 2013; Ferreira et al., 2014). Interestingly, several studies point to a cytoplasmic fraction of the Alba protein within the cell. In Plasmodium falciparum, PfAlba 1, 2 and 4 are localized as distinct cytoplasmic foci during proliferation (Chêne et al., 2012). Cytoplasmic localization of OsAlba1 was confirmed in Oryza sativa (rice), and its level of expression increased in response to dehydation stress (Verma et al., 2014). PAlba 1–PAlba 3 from Plasmodium berghei co precipitate with P-granule components PABP and eIF4E (Mair et al., 2010). Additionally, the association of TbAlba 1–TbAlba 4 with cytoplasmic mRNAs was demonstrated in Trypanosoma brucei, in which they were localized to stress granules upon starvation, indicating their role in spatiotemporal translational regulation (Mani et al., 2011; Subota et al., 2011). Similarly, the larch microsporocyte cytoplasmic Sm protein interactome was enriched in both the Alba proteins and the ribosomal and translation-related proteins.

Sm/poly(A) cytoplasmic bodies and the metabolic activity of the microsporocyte

The results presented unambiguously show that the S-bodies accumulate a large pool of mRNAs encoding different proteins. What would be the functional role of this kind of microdomain? The cytoplasm is a highly dynamic cell compartment where the antagonistic processes of mRNA translation and decay are strictly regulated (Rissland, 2017). The spatial separation of the mRNA processing, translational and degradation machinery in distinct microdomains facilitates the efficiency of post-transcriptional regulation, and protects the transcripts from uncontrolled translation or degradation, depending upon the recent needs of the cell (Parker and Sheth, 2007). In this study, we demonstrate that the S-bodies do not accumulate proteins involved in mRNA degradation and therefore are not analogs of P-bodies. We have also observed that large P-bodies are distributed in a separate area of the cytoplasm poly(A) RNA, and that the occurrence of P-bodies is most often separated temporally from periods of S-body formation. This finding is in agreement with previous observations showing that the mRNAs that accumulate within P-bodies are deadenylated (Sheth and Parker, 2003; Aizer et al., 2014), which is not the case with S-bodies.

Our previous studies have shown that the diplotene stage is the most transcriptionally active stage of prophase I (Kolowerzo-Lubnau et al., 2015). We comprehensively examined the translational activity, levels and distribution of poly(A) RNA, RNA polymerase II (an enzyme responsible for mRNA synthesis) and snRNAs, which are responsible for mRNA maturation (Smoliński et al., 2007; Smoliński et al., 2011; Smoliński and Kolowerzo, 2012; Kolowerzo-Lubnau et al., 2015; Hyjek et al., 2015). We also examined whether mRNA synthesis and maturation is accompanied by protein synthesis. The periods of intense transcription in larch microsporocytes positively correlated with periods of chromatin decondensation (the diffuse stages), whereas during the chromatin contraction stages, transcription levels decreased. As a result, RNA synthesis is not a continuous process but occurs in transcriptional bursts. The S-bodies were localized after transcriptional bursts, indicating that these microdomains are formed as a result of pulsed, extensive mRNA synthesis in the diffuse stage.

The accumulation of a vast range of mRNAs in spatially separated cytoplasmic bodies is presumably a result of the effective organization of highly expressed transcripts during periods of greatly increased export of these molecules to the cytoplasm. As S-bodies do not contain markers of degradation, the mRNAs are most likely sequestered to cope with the large number of molecules. This kind of regulation might act as a cellular strategy to control the accessibility of transcripts and their protein products during periods of genome contraction, at which time transcriptional regulation is not possible. This system enables the quick expression of proteins essential for normal cell growth and development (preparing the cell for division) and additionally provides the capability to respond to environmental changes and external stimuli. Recent studies have revealed that larch microsporocytes employ an unusual mechanism of nuclear mRNA retention, with a putative role for CBs. It was demonstrated that a significant pool of newly transcribed poly(A) RNAs is temporarily...
sequestered in the nucleoplasm and CBs before being exported to the cytoplasm (Kolowerzo et al., 2009; Smoliariski and Kolowerzo, 2012). This functional connection between nuclear mRNA retention, accumulation in CBs and cytoplasmic localization is further supported by the observation that as many as 13 of the 16 mRNAs investigated as cytoplasmic partners for Sm proteins were also localized to the CBs (see Figures 6 and S5).

**Conclusion**

In summary, S-bodies represent newly described intracellular microdomains that participate in mRNA post-transcriptional regulation. Through the temporal storage of mRNAs and their spatial separation from degradation and translation factors, these domains presumably play a role in the coordination of the continuing, frequently overlapping and competing processes of transcript maturation, translation and degradation. This strict control of mRNA fate is particularly essential in cells such as microsporocytes, in which the chromatin is periodically switched on and off and gene expression occurs in bursts. We propose a model in which the accumulation of significant quantities of different types of mRNA in cytoplasmic bodies provides a mechanism for the temporal retention and storage of highly expressed transcripts until they are needed for further translation or turnover within the cytoplasm, according to the needs of the cell. The results obtained in this work support the hypothesis that evolutionarily conserved Sm proteins have been adapted to perform a plethora of functions related to the post-transcriptional regulation of gene expression in Eukaryota. This multipurpose capacity presumably enabled them to coordinate the interdependent processes of splicing element assembly, mRNA maturation and processing, and mRNA translation regulation and degradation.

**EXPERIMENTAL PROCEDURES**

**Plant material**

For immunofluorescence/fluorescence in situ hybridization (FISH) assays, anthers of European larch (L. decidua Mill.) were collected between November and March at weekly intervals from the same tree in successive meiotic prophase (diplotene) stages to ensure consistent experimental conditions. The anthers were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 30 min, squashed to obtain free meiocytes. Meiotic protoplasts were isolated from these cells according to the method of Kolowerzo et al. (2009). Isolated protoplasts were next subjected to immunostaining, FISH and immuno-FISH assays.

For transmission electron microscopy (TEM) assays, the anthers were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 12 h and squashed to obtain free meiocytes. Meiotic protoplasts were isolated from these cells according to the method of Kolowerzo et al., 2012. This functional connection between nuclear mRNA retention, accumulation in CBs and cytoplasmic localization is further supported by the observation that as many as 13 of the 16 mRNAs investigated as cytoplasmic partners for Sm proteins were also localized to the CBs (see Figures 6 and S5).

**Immunogold labeling of Sm proteins**

Ultrathin sections were preincubated with 1% BSA in PBS, pH 7.2, for 30 min, followed by incubation with anti-Sm antibody (ANA No. 5; Centers for Disease Control and Prevention, https://www.cdc.gov) at 1:800 dilution and 4°C overnight. After washing with PBS (5 x 10 min), the samples were incubated with secondary anti-human antibody coupled to 20 nm colloidal gold particles (1:20 dilution in 1% BSA in PBS, pH 7.2; BBI Solutions, https://bbisolutions.com) for 1 h at 37°C, rinsed in PBS and water, and then contrasted with 1% phosphotungstic acid (PTA) and 2.5% uranyl acetate. The ultrastructural and immunogold analyses were performed with the use of a JEOL EM 1010 transmission electron microscope (JEOL, https://jeol.pl).

**Design of the multi-labeling reactions**

Prior to the assay, the cells were treated with 0.1% Triton X-100 in PBS for 25 min to induce cell membrane permeabilization. After labeling, the samples were stained for DNA detection with Hoechst (1:2000) and mounted in ProLong Gold Antifade reagent (Life Technologies, now ThermoFisher Scientific, https://www.thermofisher.com). The sequences for all the oligo probes used in this work are summarized in Table S1.

**Double labeling of the poly(A) RNA/U snRNA/5S rRNA and Sm proteins**

The samples were incubated with Cy3-labeled probes: oligo(dT), U1 snRNA, U2 snRNA, U4 snRNA, U5 snRNA and 5S rRNA. The 100-μm stock solutions with the probes were diluted 1:500 in hybridization buffer (30% v/v formamide, 4× saline sodium citrate (SSC), 5× Denhardt’s buffer, 1× ethylenediaminetetraacetic acid (EDTA) and 50 mM phosphate buffer) and incubated for 4 h [oligo (dT)] or overnight (other probes) at 27°C. After washing with 4× SSC (5× 1 min), 2× SSC (5× 1 min) and PBS (1× 3 min), the samples were treated with the following antibodies for the detection of the Sm proteins: human anti-Sm ANA No. 5 (1:300 in 0.2% bovine serum albumin, acetylated [acBSA] in PBS, pH 7.2), mouse anti-Sm Y12 (1:100 in 0.01% acBSA in PBS, pH 7.2; Abcam, https://www.abcam.com), mouse anti-Sm Y12 (1:500 in 0.01% acBSA in PBS, pH 7.2; a kind gift from Michael P. Terns, University of Georgia, Athens, GA, USA). The samples were then rinsed with PBS (5× 3 min) and incubated for 1 h at 37°C with the following secondary antibodies: anti-human Alexa 488 (1:750; Thermo-Fisher Scientific) or anti-mouse Alexa 488 (1:1000 in 0.01% acBSA in PBS, pH 7.2; Thermo-Fisher Scientific).

**Double labeling of the mRNA and Sm proteins**

Immunodetection of Sm proteins with ANA No. 5 was performed first to detect the mRNA and Sm proteins according to the procedure described above. The secondary anti-human tetramethylrhodamine (TRITC) antibody (1:200 in 0.01% acBSA in PBS, pH 7.2; Thermo-Fisher Scientific) was used in this assay. Next, the
samples were incubated overnight at 37°C with DIG-labeled probes that are complementary to the distinct mRNAs (Table S1) diluted 1:200 in hybridization buffer. After washing with 4× SSC (5 x 1 min), 2× SSC (5 x 1 min), 1× SSC (1 x 10 min) and PBS (1 x 3 min), the slides were incubated for 5 h at 8°C with mouse anti-digoxigenin (anti-DIG) antibody (1:200 in 0.05% acBSA in PBS, pH 7.2; Roche, https://www.roche.com) and rinsed in PBS. The samples were then incubated for 1 h at 37°C with secondary anti-mouse Alexa 488 at 1:1000 in 0.01% acBSA in PBS, pH 7.2.

**Double localization of the proteins**

Samples were incubated overnight at 4°C with the following primary antibodies: mouse anti-m3G (1:50; Santa Cruz Biotechnology, https://www.scbt.com), mouse anti-U2BP (1:20; LS Bio, https://www.lsbio.com) and rat anti-RNA pol II (elongated form, hyperphosphorylated Ser2 of the C-Terminus domain) 1:100; Chromotek, https://www.chromotek.com) in 0.01% acBSA in PBS, pH 7.2. After rinsing with PBS, the samples were incubated for 1 h at 37°C with the following secondary antibodies: anti-mouse Alexa 546 (ThermoFisher Scientific), anti-rat Alexa 488 (ThermoFisher Scientific) or anti-rabbit Alexa 488 (ThermoFisher Scientific) 1:500 in 0.01% acBSA in PBS, pH 7.2. Next, the slides were rinsed with PBS, pre-incubated with 2% BSA in PBS, pH 7.2, for 15 min, and incubated overnight at 4°C with the anti-Sm ANA No. 5 antibody diluted 1:300 in 0.2% acBSA in PBS, pH 7.2. After rinsing with PBS, the samples were incubated for 1 h at 37°C with the appropriate respective anti-human antibody: Alexa 488 at 1:750 or TRITC, at 1:200 in 0.01% acBSA in PBS, pH 7.2.

**Triple labeling of the poly(A) RNA and proteins**

For the triple-labeling assays, the oligo(dT) probe was first hybridized according to the method described above (overnight incubation). Next, the samples were incubated overnight at 4°C with the following primary antibodies: mouse anti-DCP1A (1:200; Sigma-Aldrich), rabbit anti-DCPS (1:500; a gift from Prof. Shu-Hsing Wu, Academia Sinica, Taipei, Taiwan) and rabbit anti-PAB2 (1:200; a gift from Dr Cecile Bousquet-Antonelli CNRS Perpignan Cedex, France) in 0.05% acBSA in PBS, pH 7.2. After washing with PBS, the slides were incubated for 1 h at 37°C with anti-mouse Alexa 488 Plus (1:1000) or anti-rabbit Alexa 488 Plus (1:500) in 0.01% acBSA in PBS, pH 7.2. The Sm proteins were labeled as described above with ANA No. 5 primary and anti-human Alexa 633 (1:200; ThermoFisher Scientific) secondary antibodies.

**Confocal microscopy and image analysis**

The images were captured with a Leica TCS SP8 confocal microscope at wavelengths of 405, 488, 543, 561 and 633 nm. The optimized pinhole, long exposure time (400 kHz) and a 63× (numerical aperture 1.4) Plan Apochromat DIC H oil immersion lens were used. Images were acquired sequentially in the blue (4',6-diamidino-2-phenylindole, DAPI), green (Alexa 488), red (TRITC, Cy3, Alexa 546) and/or far red (Alexa 633) channels. The optical sections were collected at 0.5-μm intervals. For the bleed-through analysis and control experiments, LAS AF software was used. For image processing and analysis, IMAGEJ software was used (Schneider et al., 2012).

For the quantitative measurements, each experiment was performed using consistent temperatures, incubation times, and concentrations of probes and antibodies. The images were collected under consistent conditions of acquisition (laser power, emission band, gain and resolution) to ensure comparable results. Before quantification of the fluorescence intensity, the background was eliminated by adjusting the threshold according to autofluorescence based on the negative control. Between 10 and 30 cells were analyzed for each stage (experimental variant), depending on the experiment. The level of fluorescence was expressed in arbitrary units (as the mean intensity per μm²).

The statistical analysis was performed using SIGMAPLOT 11.0. To compare two groups, Student’s t-tests were used. To compare between several groups, statistical significance was determined by the Kruskal-Wallis test. The significance level was set at $P < 0.05$ for all statistical tests.

Colocalization analysis was performed with the IMAGEJ plugin JA COP (Cordelieres and Bolte, 2008). Between 10 and 25 cells were analyzed for each stage (experimental variant). Only the cytoplasmic area of the cells was subject to all colocalization analyses. The colocalization coefficients were calculated (Pearson’s correlation coefficient, PC; M₁ and M₂, Manders’ overlap coefficients) for each stage and evaluated for statistical significance with Costes’ randomization test ($P < 0.05$).

The number and volume of the cytoplasmic bodies per cell were measured with IMAGEJ plugin 3D OBJECTS COUNTER (Bolte and Cordelieres, 2006). Between 10 and 25 cells for each stage (experimental variant) were analyzed. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by multiple comparisons using the Holm–Sidák post hoc test.

**Immunoprecipitation assays**

For proteomic analysis of immunoprecipitates (IP-LC-MS/MS), contents from the cytoplasmic fraction of the cells were immunoprecipitated according to a modified protocol based on that used by Oliva et al. (2016). Briefly, larch anthers were ground in liquid nitrogen and homogenized in protein extraction buffer at 3 ml per 1 g of plant material. The homogenates were then centrifuged twice at 14,000 g, followed by centrifugation at 16,000 g for 30 min at 4°C, to obtain native cytosolic protein extract. The supernatant (cytosolic fraction) was pre-cleared with MagneticNiP Ag/G magnetic beads (Merck, https://www.merckmillipore.com) for 1 h at 4°C and incubated with anti-Sm Y12 antibody (a kind gift from Michael P. Terns, University of Georgia, Athens, GA, USA) overnight at 4°C. Next, a fresh set of magnetic beads was added, and the mixture was incubated for 1 h at 4°C. The beads were precipitated by immobilization on a magnetic separator (ThermoFisher Scientific), washed four times with PBST (0.1%, Tween 20) and resuspended in water. The experiment was performed in quadruplicate for the IP assay and triplicate for the mock IP assay (no antibody added). The LC-MS/MS analysis was performed by the Mass Spectrometry Laboratory (IBB PAS, Warsaw, Poland) with the use of an Orbitrap mass spectrometer (ThermoFisher Scientific). The proteins were identified with MASCOT (Matrix Science, http://www.matrixscience.com) and the TAIR10 database (The Arabidopsis Information Resource, TAIR, https://www.arabidopsis.org). Next, the samples were analyzed with MASCAN software (http://proteom.ibr.ibb.waw.pl/mscan/index.html). The list of Sm protein-immunoprecipitated proteins was corrected with the results from the mock IP experiments. The protein was considered Sm protein-immunoprecipitated when it was present in at least three IP samples and not present in at least two control (mock IP) samples.

For the RNA immunoprecipitation (RIP) from the cytoplasmic fraction, the assays were performed according to a modified protocol from Sorenson and Bailey-Serres (2015). Briefly, larch anthers were ground in liquid nitrogen and RNP extraction buffer (200 mM Tris-HCl, pH 9.0, 110 mM potassium acetate, 0.5% (v/v) Triton X-100, 0.1% (v/v) Tween 20, 2.5 mM dithiothreitol (DTT), complete protease inhibitor (Roche) and 0.04 U µl⁻¹ RNase...
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with BLASTX (for putative mRNAs) and BLASTN (for other RNAs) with the NCBI, https://www.ncbi.nlm.nih.gov) nr and nt databases, respectively. The use of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) nr and nt databases, respectively. Consensus functions and ontologies were found by LCA (least common ancestor) methodologies implemented in BLAST2GO 4.1 (Götz et al., 2008).

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AUTHOR CONTRIBUTIONS

MSH participated in experimental design, performed the majority of experiments and participated in writing the article. MB and PN performed immunoprecipitation assays and cDNA library preparation. MG performed bioinformatic analysis and participated in the discussion, analysis of results and writing of the article. AKL performed confocal microscopy and image analysis and participated in discussion. AJ designed experiments and participated in the discussion and analyses of the results. DJS performed immunogold labeling, double localization of the proteins, triple labeling of the RNA and proteins, confocal microscopy and image analysis, designed experiments, participated in the discussion and analyses of the results, and in the writing of the article.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this work are provided in the main text and the supporting information. Raw reads generated during this project were deposited in the NCBI SRA database and are accessible through BioProject no. PRJNA624007. The full data set for the transcriptomic analysis is available at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA624007. All other data and materials used in this study will be available at request from the corresponding authors.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Western blot analysis of protein extracts obtained after immunoprecipitation using different types of anti-Sm antibodies: Y12, ANA No. 5 and Y12 (Abcam).

Figure S2. Identification of Sm Larix decidua proteins by IP-SDS-PAGE-MS.

Figure S3. Localization of the Sm proteins and poly(A) RNA on semi-thin sections of Larix decidua Mill. anther.

Figure S4. Labeling of Sm proteins and selected splicing U snRNAs in larch microsporocytes.

Figure S5. Localization of mRNA precipitating with Sm proteins in situ.

Figure S6. Localization of the Sm mRNAs and Sm proteins in situ.

Figure S7. Validation of the Sm mRNAs and Sm proteins localization with TruSeq Stranded Total RNA with a Ribo-Zero Plant kit (Schmieder and Edwards, 2011). The sequence stretches with an average quality of <20 over a window of 20 nt were removed, and the reads were cut immediately before the first incidence of a degenerated base. Furthermore, trimmed reads shorter than 36 nt were removed. The reads from all libraries were pooled and assembled de novo using Trinity 2.8.2 with default settings (Grabherr et al., 2011). The transcript sequences were next analyzed with BLASTX (for putative mRNAs) and BLASTN (for other RNAs) with the use of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) nr and nt databases, respectively. Consensus functions and ontologies were found by LCA (least common ancestor) methodologies implemented in BLAST2GO 4.1 (Götz et al., 2008).

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SMH participated in experimental design, performed the majority of experiments and participated in writing the article. MB and PN performed immunoprecipitation assays and cDNA library preparation. MG performed bioinformatic analysis and participated in the discussion, analysis of results and writing of the article. AKL performed confocal microscopy and image analysis and participated in discussion. AJ designed experiments and participated in the discussion and analyses of the results. DJS performed immunogold labeling, double localization of the proteins, triple labeling of the RNA and proteins, confocal microscopy and image analysis, designed experiments, participated in the discussion and analyses of the results, and in the writing of the article.

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Figure S7. Validation of the Larix decidua anther cell fractionation method.

Table S1. Real-time RT-PCR primers and probe sequences used for in situ hybridization.
Table S2. Identification of the proteins that form a complex with Sm proteins in the cytoplasm determined by IP-LC-MS/MS.

Table S3. The cytoplasmic Sm protein interactome.

Appendix S1. List of Sm protein-associated transcripts and sequences determined by RIP-seq.

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