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Regulación post-transcripcional del desarrollo y la respuesta a estrés abiótico de *Arabidopsis thaliana* L. mediada por el complejo LSM1-7

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Carlos Perea Resa

Director

Julio Salinas Muñoz

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Memoria presentada por Carlos Perea Resa, inscrito en el programa de doctorado del Departamento de Genética de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid, para optar al grado de Doctor en Ciencias Biológicas.

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VºBº del Director

Carlos Perea Resa

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“Es genial comprobar que todavía tienes la capacidad de sorprenderte a ti mismo”

Kevin Spacey, American Beauty.

ÍNDICE

1. INTRODUCCIÓN

Los organismos superiores están compuestos por un gran número de células diferenciadas, resultado de un complejo programa de desarrollo así como de constantes interacciones con el medio ambiente que les rodea. Las plantas, como organismos sésiles, han adquirido además, a lo largo de la evolución, diversos mecanismos que les permiten adecuar su desarrollo y fisiología a condiciones ambientales desfavorables. La expresión génica y su regulación juegan un papel esencial en la diferenciación celular en plantas, así como en su rápida y eficiente adaptación a cambios ambientales. Estudios globales han puesto de manifiesto el importante grado de complejidad y especificidad en los patrones de expresión de miles de genes a lo largo del desarrollo de *Arabidopsis thaliana* L. (1-3). Además, han revelado la extensa reprogramación que dicha expresión experimenta en respuesta a estreses de carácter abiótico (4-6). Estos ajustes están regulados en gran medida a nivel transcripcional (7-10), aunque en los últimos años, sin embargo, numerosos trabajos indican que la regulación a nivel post-transcripcional también juega un papel importante (11-13).

La regulación de la expresión a nivel post-transcripcional posibilita ajustes rápidos en los niveles de mensajeros funcionales de manera independiente a su síntesis *de novo*. En particular, la degradación de mRNAs resulta crucial para el control de su vida media, lo que en última instancia determina su capacidad para ser traducidos. El proceso de degradación, extensamente estudiado en levaduras (14), es llevado a cabo principalmente en el citoplasma y comienza con la digestión de la cola de poli (A) presente en el extremo 3´ del mRNA, un proceso controlado por el complejo CARBON CATABOLITE REPRESSOR 4/ CCR4 ASSOCIATED FACTOR-1 (CCR4-CAF1) (15, 16). A continuación, la mayoría de los mensajeros sufren la escisión de la caperuza presente en su extremo 5´ (5´cap) por medio de la maquinaria de *decapping* compuesta esencialmente por DECAPPING 1 (DCP1) y DECAPPING 2 (DCP2), siendo esta última la subunidad en la que reside la actividad enzimática (17, 18). Finalmente, los tránscritos sin estructura 5´ cap son susceptibles de ser degradados en dirección 5´-3´ mediante la acción de la exonucleasa EXORIBONUCLEASE 1 (XRN1) (19). La acción de todos estos factores es llevada a cabo en sitios concretos del citoplasma denominados cuerpos de procesamiento (*P-bodies*), en donde mRNAs y factores de degradación confluyen (20, 21). Alternativamente, una vez los mensajeros son deadenilados, estos pueden ser degradados en dirección 3´-5´ por un complejo multiproteico denominado Exosoma (22). En plantas, han sido identificados factores homólogos a componentes de ambas rutas de degradación, demostrando la conservación evolutiva de este proceso en el reino vegetal. Sin embargo, estudios sobre la contribución y especificidad de estas dos vías mayoritarias sugieren un escenario particular (23). Así, en Arabidopsis, distintos estudios funcionales han determinado que CCR4-CAF1 y POLY (A) RIBONUCLEASE (PARN) poseen actividad deadenilasa. No obstante, la implicación de la POLY (A) NUCLEASE (PAN) en la deadenilación aún no ha sido demostrada (24). Análogamente, componentes del complejo de *decapping* fueron descritos de manera independiente por tres grupos (25-27), estableciéndose en todos los casos que la actividad de DCP2 es facilitada por DCP1 y VARICOSE (VCS), componentes esenciales para la funcionalidad del complejo. A continuación, la identificación de DECAPPING 5 (DCP5) y PROTEIN ASSOCIATED TO TOPOISOMERASE II 1 (PAT1), dos nuevos activadores del *decapping*, evidenció la complejidad de la maquinaria encargada de este proceso en plantas (28, 29). Finalmente, la EXORIBONUCLEASE 4 (XRN4), proteína citoplásmica con actividad exonucleasa, actuaría de manera homóloga a la enzima XRN1 de levaduras completando el proceso de degradación del mRNA (30, 31). Numerosos trabajos han establecido el importante papel que tiene la degradación de mensajeros en el control de la expresión génica durante el desarrollo (25-28, 32, 33). Sin embargo, tan solo recientemente se ha descrito su implicación en la reprogramación que dicha expresión experimenta en respuesta a condiciones de estrés (34-36).

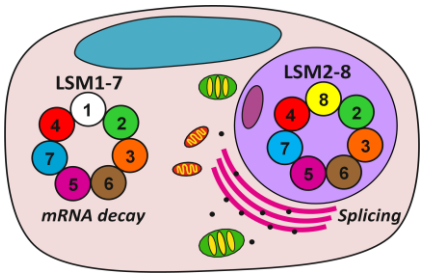

Figure1. Esquema representativo de los complejos LSM dentro una célula eucariota. Se indican los componentes de cada complejo, su localización subcelular y el principal proceso del metabolismo de RNA en el cual participan.

Las proteínas SM-like (LSM) son proteínas de bajo peso molecular encuadradas en la misma familia que las proteínas SM, identificadas por vez primera en el suero de un enfermo de Lupus Eritematoso Sistémico y que deben su nombre al apellido de dicho paciente (Stephanie Smith) (37). Componentes de esta familia han sido encontrados en especies eucariotas, en bacterias y en arqueobacterias, incluso se conjetura la posibilidad de que estuvieran presentes en el genoma del último ancestro común universal (LUCA) (38), demostrando su relevancia a lo largo de la evolución. En levaduras, han sido descritas ocho proteínas LSM altamente conservadas (LSM1-8) definidas por su particular estructura tridimensional, la cual les permite interaccionar entre ellas formando complejos heteroheptaméricos con forma de anillo que participan en diversos aspectos del metabolismo de

RNAs. Existen dos complejos LSM con diferente localización subcelular y función (Figura 1). El complejo nuclear LSM2-8 participa en el procesamiento de pre-mRNAs interaccionando y estabilizando el *small nuclear* RNA U6, componente esencial del espliceosoma (39). Por otro lado, el complejo citoplásmico LSM1-7 participa en la degradación 5´-3´ de mensajeros que tiene lugar en los *P-bodies* mediante la unión directa a su extremo 3´ una vez completado el proceso de deadenilación (40). Esta unión, específica de mensajeros oligoadenilados, origina la protección del mRNA frente a la degradación 3´-5´ mediada por el Exosoma (41, 42), promueve el reclutamiento y la actividad de los componentes del complejo de *decapping* y, finalmente, la posterior degradación mediada por la exonucleasa XRN1 (40). Dentro del complejo citoplásmico se ha establecido que la proteína LSM1, además de ser la subunidad que lo define y caracteriza, es esencial para su formación (43, 44), para la unión específica al mensajero oligoadenilado (42), y para la integridad de los *P-bodies* (45). La secuenciación del genoma de Arabidopsis y su posterior anotación permitió identificar once genes homólogos *LSM* (46). Sin embargo, su caracterización, y la de las correspondientes proteínas LSM, todavía no ha sido llevada a cabo.

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2. OBJETIVOS

Teniendo en cuenta los antecedentes descritos, en esta tesis nos planteamos los siguientes objetivos:

1. Identificación y caracterización molecular del complejo citoplásmico LSM1-7 de Arabidopsis.

2. Caracterización funcional del complejo LSM1-7 en el desarrollo de Arabidopsis y en su adaptación a condiciones de estrés abiótico.

3. RESULTADOS

Los resultados obtenidos en torno al primero de los objetivos se presentan y discuten íntegramente en un trabajo publicado en la revista *The Plant Cell* (pag.5). En esta misma publicación se describe el papel esencial que el complejo LSM1-7 juega en la regulación del desarrollo de Arabidopsis. Por otro lado, el conjunto de resultados en torno a la caracterización del complejo citoplásmico en la respuesta y adaptación de Arabidopsis a situaciones de estrés abiótico, se presentan en un segundo trabajo recientemente enviado para su publicación en la revista *Molecular Cell* (pag.24). Además, como resultado del estudio de la implicación del complejo LSM1-7 en la respuesta de Arabidopsis a las temperaturas bajas, se desarrolló un método para evaluar la tolerancia a las heladas de mutantes de Arabidopsis (pag.37). Este protocolo fue publicado en un capítulo del libro titulado *Plant Cold Acclimation: Methods and Protocols*, perteneciente a la serie *Methods in Molecular Biology* editada por *Springer Science*, y también forma parte de la presente memoria.

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LSM Proteins Provide Accurate Splicing and Decay of Selected Transcripts to Ensure Normal *Arabidopsis* Development[W]

Carlos Perea-Resa,[1] Tamara Hernández-Verdeja,[1,2] Rosa López-Cobollo,[3] María del Mar Castellano,[2] and Julio Salinas[4]

Departamento de Biología Medioambiental, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain

In yeast and animals, SM-like (LSM) proteins typically exist as heptameric complexes and are involved in different aspects of RNA metabolism. Eight LSM proteins, LSM1 to 8, are highly conserved and form two distinct heteroheptameric complexes, LSM1-7 and LSM2-8,that function in mRNA decay and splicing, respectively. A search of the *Arabidopsis thaliana* genome identifies 11 genes encoding proteins related to the eight conserved LSMs, the genes encoding the putative LSM1, LSM3, and LSM6 proteins being duplicated. Here, we report the molecular and functional characterization of the *Arabidopsis LSM* gene family. Our results show that the 11 *LSM* genes are active and encode proteins that are also organized in two different heptameric complexes. The LSM1-7 complex is cytoplasmic and is involved in P-body formation and mRNA decay by promoting decapping. The LSM2-8 complex is nuclear and is required for precursor mRNA splicing through U6 small nuclear RNA stabilization. More importantly, our results also reveal that these complexes are essential for the correct turnover and splicing of selected development-related mRNAs and for the normal development of *Arabidopsis*. We propose that LSMs play a critical role in *Arabidopsis* development by ensuring the appropriate development-related gene expression through the regulation of mRNA splicing and decay.

INTRODUCTION

During the last years, an increasing body of evidence indicates that posttranscriptional regulation plays an important role in modulating gene expression during development in eukaryotes (Halbeisen et al., 2008). Most eukaryotic genes are transcribed as precursors (pre-mRNAs) containing intron sequences. In order to yield correct translation products, introns need to be excised to generate mature mRNAs. This process, known as pre-mRNA splicing, is fundamental in both constitutive and regulated gene expression. Pre-mRNA splicing is precisely and efficiently performed by the spliceosome, a large ribonucleoprotein (RNP) complex machinery composed of five small nuclear RNP particles (U1, U2, U4/U6, and U5) and more than 200 polypeptides not tightly associated with snRNPs (Wahl et al., 2009). In many cases, however, the splicing process is flexible enough to allow the generation of alternative transcripts from a single gene by differential use of splicing sites. Site use may depend on the cell type, developmental stage, or physiological condition, thereby affecting protein diversity and transcript levels (Matlin et al., 2005). The general mechanism of splicing has been well studied in humans and yeast, being largely conserved between these organisms. In plants, the splicing process remains comparatively poorly understood, although the basic mechanisms of spliceosome assembly and intron excision appear to be as in the rest of eukaryotes (Lorković et al., 2000; Reddy, 2001). Consistent with this, the analysis of the *Arabidopsis thaliana* genome for the presence of known spliceosomal proteins indicated that the core of spliceosomal machinery is conserved between plants and animals (Wang and Brendel, 2004). Nonetheless, despite this conservation, incorrect splicing of mammalian pre-mRNAs in plant cells and vice versa denotes the existence of plant-specific splicing regulatory mechanisms requiring plant-specific splicing factors (Lorković et al., 2000; Reddy, 2001; Lorković, 2009). The characterization of different plant splicing proteins, including some Gly-rich RNA binding proteins, SR proteins, RNA helicases, and other RNA binding proteins, have revealed that they are essential for the accurate progress of diverse plant developmental processes (Raab and Hoth, 2007; Barta et al., 2008; Lorković, 2009; Deng et al., 2010; Zhang et al., 2011).

The control of mRNA turnover is another critical aspect in the regulation of eukaryotic gene expression. Two major pathways exist in yeast and mammals for mRNA decay, both of them being initiated by deadenylation through the CARBON CATABOLITE REPRESSION4/PGK PROMOTER DIRECTED OVERPRODUCTION2/NEGATIVE ON TATA-1 complex (Meyer

[1] These authors contributed equally to this work.
[2] Current address: Centro de Biotecnología y Genómica de Plantas Universidad Politécnica de Madrid/Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Campus de Montegancedo, 28223 Madrid, Spain.
[3] Current address: Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom.
[4] Address correspondence to salinas@cib.csic.es.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Julio Salinas (salinas@cib.csic.es).
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et al., 2004; Parker and Song, 2004). Subsequently, transcripts can be processed by the 3′ to 5′ or the 5′ to 3′ decay pathways. In the first pathway, the deadenylated mRNA is degraded by a complex of proteins known as the exosome (Anderson and Parker, 1998). In the second pathway, the mRNA is decapped by the mRNA DECAPPING1 (DCP1)/DCP2 enzyme, making the mRNA susceptible to the EXORIBONUCLEASE1 (XRN1) (Beelman et al., 1996; Dunckley and Parker, 1999). Therefore, decapping is an important node in the regulation of mRNA lifespan and is modulated by a set of different proteins (Bonnerot et al., 2000; Coller et al., 2001). The decapping machinery accumulates in discrete cytoplasmic foci named processing bodies (P-bodies), which have been suggested to be functionally involved not only in mRNA decapping (Sheth and Parker, 2003; Cougot et al., 2004) but also in nonsense-mediated mRNA decay (Unterholzner and Izaurralde, 2004; Sheth and Parker, 2006), mRNA storage (Brengues et al., 2005), general translation repression (Coller and Parker, 2005), and microRNA-mediated repression (Bhattacharyya et al., 2006). Although the existence of both the 5′ to 3′ and the 3′ to 5′ decay pathways has been documented and their core components identified (Xu et al., 2006; Goeres et al., 2007; Belostotsky and Sieburth, 2009; Lange and Gagliardi, 2010), the governing principles of mRNA decay in plants, as in the case of the splicing process, are still poorly known. Moreover, genetic analyses have also uncovered plant-specific functional features in mRNA degradation pathways that are associated with plant-specific factors (Belostotsky and Sieburth, 2009; Xu and Chua, 2011). In *Arabidopsis*, for instance, no *XRN1*-like gene has been identified. Instead, the cytoplasmic 5′ to 3′ exoribonuclease activity is performed by XRN4 (Kastenmayer and Green, 2000; Souret et al., 2004). Plant P-bodies seem to function as yeast and human P-bodies. However, they also contain their own distinct protein components (Xu and Chua, 2011). Plants affected in mRNA turnover display severe developmental perturbations, indicating that proteins related to mRNA decapping and decay play important roles in regulating gene expression during plant development (Xu et al., 2006; Goeres et al., 2007; Belostotsky and Sieburth, 2009; Xu and Chua, 2009, 2011).

The SM-like proteins (LSMs) constitute a large family of proteins that function in multiple aspects of RNA metabolism. In yeast and animals, there are eight highly conserved LSM proteins (LSM1 to LSM8) that form two different heptameric ring complexes, LSM1-7 and LSM2-8, localized in the cytoplasm and nucleus, respectively. LSM1 and LSM8 define and confer the specificity to each complex, while the other proteins (LSM2 to LSM7) participate in both cytoplasmic and nuclear complexes. The LSM1-7 cytoplasmic complex binds to oligoadenylated mRNAs, promoting their decapping and subsequent degradation by the 5′ to 3′ pathway, and accumulates in P-bodies. The LSM2-8 nuclear complex binds to and stabilizes the U6 small nuclear RNA (snRNA), forms the core of the U6 small nuclear RNP, and functions in pre-mRNA splicing (reviewed in Beggs, 2005; Tharun, 2009). In silico approaches have allowed the identification of potential plant homologs of LSM proteins. *Arabidopsis* has homologs for the eight conserved LSMs, and three of them (LSM1, LSM3, and LSM6) are duplicated (Wang and Brendel, 2004). To date, however, plant LSMs have not been functionally characterized, and their role in RNA metabolism remains to be determined. Only *Arabidopsis LSM5* and *LSM4* genes have been experimentally studied, both of them being related to abscisic acid and osmotic stress signaling (Xiong et al., 2001; Deng et al., 2010; Zhang et al., 2011). Here, we report the molecular and functional characterization of the *Arabidopsis LSM* gene family. Our results indicate that *Arabidopsis* LSM proteins are also organized in two different heptameric complexes localized in the cytoplasm and nucleus. Whereas the cytoplasmic complex (LSM1-7) is involved in P-body formation, mRNA decapping, and, therefore, accurate mRNA decay, the nuclear complex (LSM2-8) is required for U6 snRNA stabilization and, consequently, proper pre-mRNA splicing. Genetic and molecular analyses reveal that LSM1-7 and LSM2-8 complexes are essential for the correct turnover and splicing of selected development-related mRNAs, respectively. Consistent with this, the absence of LSM1 and LSM8 proteins causes severe perturbations in *Arabidopsis* deveopment, which correlates with alterations in developmentally regulated gene expression. We conclude that LSMs play a critical role in *Arabidopsis* development by ensuring the appropriate development-related gene expression through the regulation of mRNA splicing and decay.

RESULTS

The *Arabidopsis* Genome Contains 11 Genes Encoding the Eight Highly Conserved LSM Proteins

Sequence comparisons and motif searches allowed the identification of 11 genes in the *Arabidopsis* genome encoding proteins related to the eight highly conserved proteins that in yeast and animals constitute the heptameric LSM complexes, LSM1-7 and LSM2-8, with three of them, the putative *LSM1*, *LSM3*, and *LSM6*, being duplicated (Wang and Brendel, 2004). The predicted *Arabidopsis* proteins contain the Sm1 and Sm2 motifs that are separated by a nonconserved linker region of variable length and conform the Sm bipartite domain typical of LSM proteins (Tharun, 2009) (see Supplemental Figure 1 online). LSM proteins have also been found in the genomes of different plant species (Proost et al., 2009; Goodstein et al., 2012). A phylogenetic analysis was performed with the LSM proteins from *Arabidopsis* and other representative plant species, including soybean (*Glycine max*), poplar (*Populus trichocarpa*), rice (*Oryza sativa*), and maize (*Zea mays*). Results revealed that all plant genomes analyzed contain genes encoding LSMs related to the eight conserved proteins from yeast and animals and that many of them are present in more than one copy as in the case of *Arabidopsis LSM1*, *LSM3*, and *LSM6*. The human LSM proteins were also included in the analysis as an internal control (see Supplemental Figure 2 and Supplemental Data Set 1 online).

RNA gel blot analysis revealed that the 11 *Arabidopsis LSM* genes are expressed in all organs tested, including leaves, roots, flowers, and stems. Each pair of duplicated genes exhibited the same expression pattern (Figure 1A). To determine the expression of *LSM* genes at the tissue level, transgenic *Arabidopsis* plants containing fusions between all *LSM* promoters (LSM_{pro})

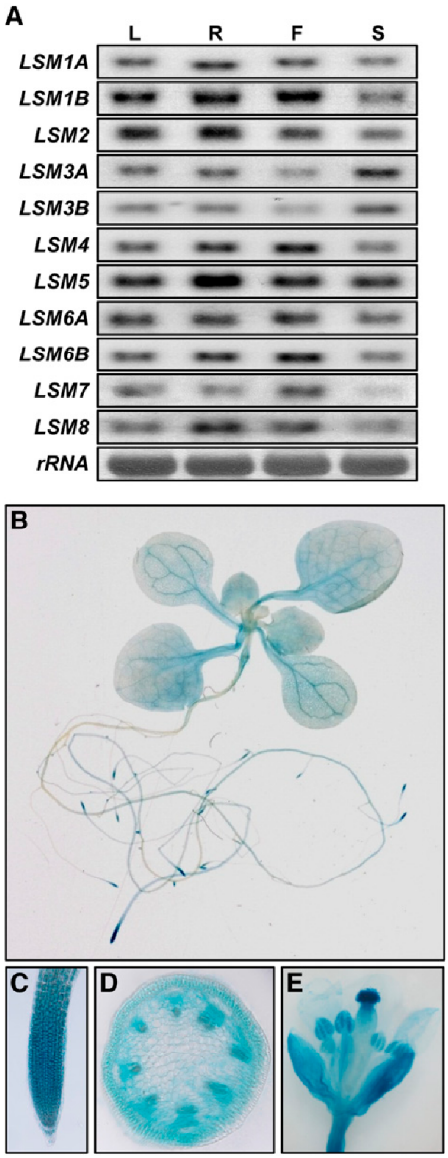

Figure 1. Expression Patterns of *Arabidopsis LSM* Genes.

(A) Expression analysis of *LSM* genes in different organs of *Arabidopsis* by RNA hybridization using specific probes. Total RNA (20 μg) from 4-week-old rosette leaves (L), roots (R), flowers (F), and stems (S) was used. *rRNA* levels are shown as a loading control.

(B) to **(E)** GUS activity in *Arabidopsis* plants containing the fusion $LSM8_{pro}$-*GUS*. Whole plant **(B)**, root **(C)**, cross section of a stem **(D)**, and flower **(E)**.

and the β-glucuronidase (*GUS*) reporter gene were generated and assayed for GUS activity. Consistent with the results obtained from the RNA gel blot experiments, nearly constitutive GUS activity was observed in all cases. In leaves and cotyledons, GUS staining was preferentially detected in the vascular tissues. As representative examples, the expression of $LSM8_{pro}$-*GUS*, $LSM1A_{pro}$-*GUS*, and $LSM1B_{pro}$-*GUS* is shown (Figures 1B to 1E; see Supplemental Figure 3 online). These results demonstrate that the 11 *Arabidopsis LSM* genes are active and ubiquitously expressed.

Subcellular Localization of *Arabidopsis* LSM Proteins

To investigate the subcellular localization of *Arabidopsis* LSM proteins, transgenic *Arabidopsis* expressing genomic *LSM*–green fluorescent protein (*LSM-GFP*) fusions driven by the corresponding LSM_{pro} were obtained and analyzed. We first examined the subcellular localization of LSM1A, LSM1B, and LSM8, the *Arabidopsis* putative homologs of yeast and animal LSM proteins that differentiate the cytoplasmic and nuclear complexes, respectively. In root cells from seedlings expressing $LSM1A_{pro}$-*LSM1A-GFP* or $LSM1B_{pro}$-*LSM1B-GFP*, green fluorescence suggested a cytoplasmic localization of LSM1A and LSM1B (Figure 2A). Conversely, in seedlings expressing the $LSM8_{pro}$-*LSM8-GFP* fusion, green fluorescence was specifically localized in nuclei (Figure 2A). We also investigated the subcellular localization of *Arabidopsis* LSM3A, LSM3B, and LSM4, whose related yeast and animal proteins participate in both cytoplasmic and nuclear LSM complexes. In root cells from seedlings expressing $LSM3A_{pro}$-*LSM3A-GFP* or $LSM3B_{pro}$-*LSM3B-GFP*, green fluorescence was detected in both nuclei and cytoplasm, indicating that LSM3A and LSM3B proteins simultaneously localize to these subcellular compartments (Figure 2A). Similar results were obtained when studying the subcellular localization of the LSM4-GFP fusion protein in seedlings expressing $LSM4_{pro}$-*LSM4-GFP* (Figure 2A). These data strongly suggest that *Arabidopsis* LSM proteins have subcellular localizations similar to the LSM proteins from other eukaryotes.

Yeast and human LSM1-7 proteins have been described to accumulate in P-bodies (Ingelfinger et al., 2002; Sheth and Parker, 2003). We therefore examined whether *Arabidopsis* LSM proteins belonging to the cytoplasmic complex also localized in these cytoplasmic foci. P-bodies are rarely observed in plants growing under control conditions, whereas their number and size markedly increase under conditions that are associated with high levels of mRNA turnover, such as hypoxic or heat stress (Weber et al., 2008). When seedlings expressing $LSM1A_{pro}$-*LSM1A-GFP* or $LSM1B_{pro}$-*LSM1B-GFP* were exposed to heat stress, LSM1A-GFP and LSM1B-GFP were largely localized to discrete cytoplasmic spots (Figure 2B). Under heat stress conditions, LSM3A-GFP, LSM3B-GFP, and LSM4-GFP fusion proteins also localized to cytoplasmic foci in root cells from seedlings expressing $LSM3A_{pro}$-*LSM3A-GFP*, $LSM3B_{pro}$-*LSM3B-GFP*, or $LSM4_{pro}$-*LSM4-GFP*, respectively (Figure 2B). Following cycloheximide treatment, which in yeast and humans results in the loss of P-bodies (Sheth and Parker, 2003), no cytoplasmic foci were observed in any case (Figure 2C), suggesting that the detected cytoplasmic spots of LSM-GFP fusion proteins corresponded to P-bodies. Consistent with its specific nuclear localization, LSM8-GFP did not accumulate in cytoplasmic spots in $LSM8_{pro}$-*LSM8-GFP* seedlings exposed to heat stress (Figure 2B) or to heat stress plus cycloheximide (Figure 2C). To confirm that the foci defined by *Arabidopsis* cytoplasmic LSM proteins corresponded to P-bodies, we further analyzed their colocalization with DCP1,

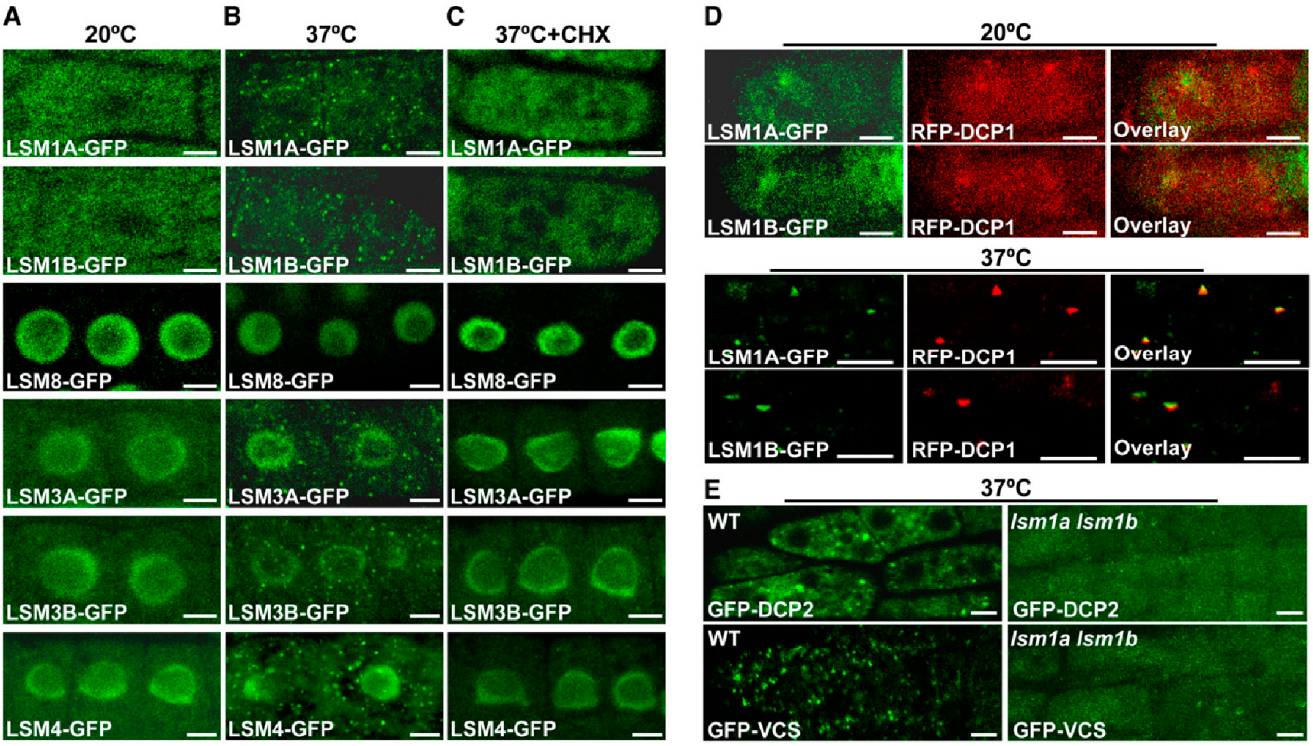

Figure 2. Subcellular Localization of *Arabidopsis* LSM Proteins.

(A) to **(C)** Subcellular localization of different LSM-GFP proteins in root tip cells from 6-d-old *Arabidopsis* seedlings. Seedlings grown under control conditions **(A)**, seedlings grown under control conditions and subsequently exposed 2 h at 37°C **(B)**, and seedlings grown under control conditions and subsequently exposed 2 h at 37°C with cycloheximide (CHX) **(C)**. Bars = 10 μm.

(D) Colocalization of LSM1A-GFP and LSM1B-GFP with RFP-DCP1 in root tip cells from 6-d-old *Arabidopsis* seedlings grown under control conditions (top panel) and subsequently exposed 2 h at 37°C (bottom panel). Bars = 10 μm.

(E) Subcellular localization of GFP-DCP2 and GFP-VCS in root tip cells from 6-d-old wild-type (WT) and *lsm1a lsm1b Arabidopsis* seedlings grown under control conditions and subsequently exposed 2 h at 37°C. Bars = 10 μm.

a protein that belongs to the *Arabidopsis* decapping complex and accumulates in P-bodies (Xu et al., 2006; Goeres et al., 2007). The examination of seedlings expressing *LSM1A*$_{pro}$*-LSM1A-GFP* or *LSM1B*$_{pro}$*-LSM1B-GFP* cotransformed with a *35S*–red fluorescent protein (*RFP*)*–DCP1* fusion revealed that, in fact, LSM1A-GFP and LSM1B-GFP colocalized with RFP-DCP1 in root cells grown at room temperature (20°C) or exposed to 37°C (Figure 2D). Taken together, these observations demonstrate that *Arabidopsis* cytoplasmic LSM proteins accumulate in P-bodies.

DCP2 and VARICOSE (VCS), like DCP1, also belong to the *Arabidopsis* decapping complex and accumulate in P-bodies (Xu et al., 2006; Goeres et al., 2007). Accordingly, GFP-DCP2 and GFP-VCS fusion proteins localized to P-bodies within the cytoplasm of root cells from wild-type seedlings containing *35S-GFP-DCP2* or *35S-GFP-VCS* constructs, respectively, exposed to heat stress (Figure 2E). When these constructs were introduced into an *Arabidopsis* mutant defective in LSM1 proteins (*lsm1a lsm1b*; see below) under the same stress conditions, the P-bodies were lost and the GFP-DCP2 and GFP-VCS signals were mostly dispersed in the cytosol (Figure 2E). From these results we conclude that, in addition to accumulating in P-bodies, LSM1 proteins are required for P-body formation in *Arabidopsis*.

Organization of *Arabidopsis* LSM Proteins

As mentioned above, yeast and animal LSM proteins typically exist as highly organized ring-shaped heptameric complexes (Figure 3A) (Tharun, 2009). Having established that *Arabidopsis* LSM proteins subcellularly localize as in other eukaryotes, we decided to study how they are organized. For this, we assayed in vivo LSM–LSM interactions by means of bimolecular fluorescence complementation (BiFC) (Hu et al., 2002; Walter et al., 2004) in *Nicotiana benthamiana* leaves. In yeast and animal LSM complexes, LSM1 and LSM8 are flanked by LSM2 and LSM4 (Figure 3A). Our experiments revealed that a significant proportion of cells cotransformed with *LSM1A-nGFP* and *LSM2-cGFP* or *LSM4-cGFP*, and *LSM8-nGFP* and *LSM2-cGFP* or *LSM4-cGFP* displayed green fluorescence (Figure 3B). Identical results were obtained cotransforming *LSM1B-nGFP* with *LSM2-cGFP* or *LSM4-cGFP* (see Supplemental Figure 4 online), indicating that *Arabidopsis* LSM1A, LSM1B, and LSM8 are capable of interacting in vivo with LSM2 and LSM4. Consistent with the typical cytoplasmic localization of LSM1 proteins in *Arabidopsis* (Figure 2A), LSM1(A or B)–LSM2 and LSM1(A or B)–LSM4 interactions mainly appeared in the cytoplasm of the *N. benthamiana* cells (Figure 3B; see Supplemental Figure 4 online).

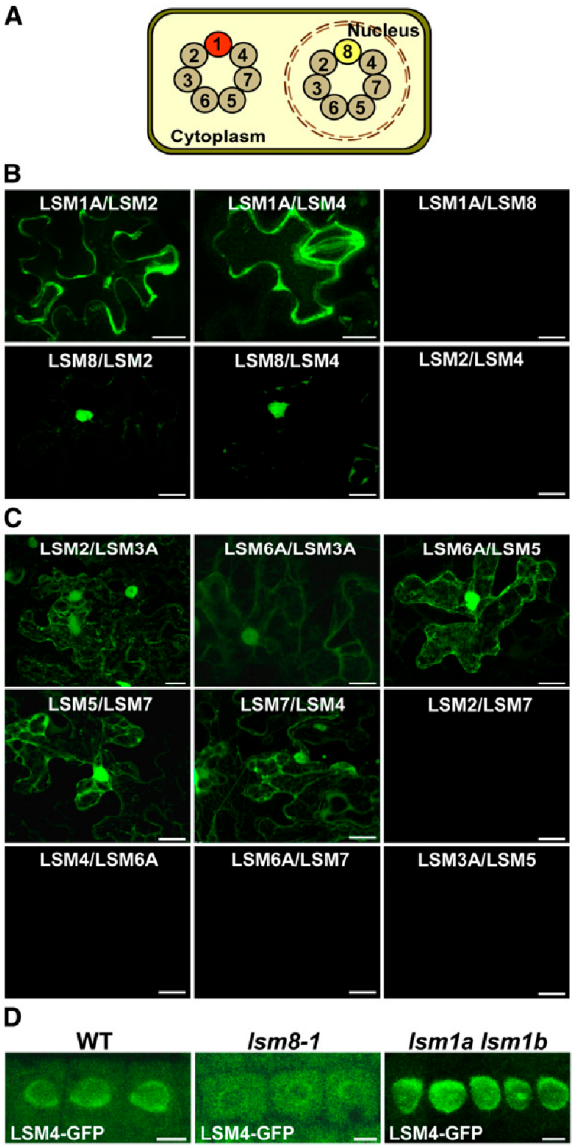

Figure 3. Organization of *Arabidopsis* LSM Proteins.

(A) Cellular model showing cytoplasmic and nuclear heptameric LSM complexes as described in yeast and humans.
(B) and **(C)** Visualization of in vivo interactions between *Arabidopsis* LSM proteins by BiFC assays. The corresponding LSM-nGFP/LSM-cGFP proteins were pairwise tested by *Agrobacterium tumefaciens*–mediated transformation in *N. benthamiana* leaves. Interactions between LSM1A/LSM2, LSM1A/LSM4, LSM1A/LSM8, LSM8/LSM2, LSM8/LSM4, and LSM2/LSM4 **(B)**, and LSM2/LSM3A, LSM6A/LSM3A, LSM6A/LSM5, LSM5/LSM7, LSM7/LSM4, LSM2/LSM7, LSM4/LSM6A, LSM6A/LSM7, and LSM3A/LSM5 **(C)** are presented. Bars = 20 μm.
(D) Subcellular localization of LSM4-GFP in root tip cells from 6-d-old wild-type (WT), *lsm8-1*, and *lsm1a lsm1b Arabidopsis* seedlings grown under control conditions. Bars = 10 μm.

Conversely, interactions between LSM8 and LSM2 and LSM4 were essentially detected in the nucleus (Figure 3B), which is consistent with the characteristic nuclear localization of *Arabidopsis* LSM8 protein (Figure 2A). The specificity of all these interactions was demonstrated by the fact that, as expected from their different subcellular localization, we did not observe interaction between LSM1 proteins and LSM8 (Figure 3B; see Supplemental Figure 4 online). Interactions between LSM2 and LSM4 proteins were not found either (Figure 3B), in agreement with what has been proposed for yeast and animal LSM complexes (Figure 3A) (Beggs, 2005). However, we detected interactions between LSM2 and LSM3 (A or B), LSM3 (A or B), and LSM6 (A or B); LSM6 (A or B) and LSM5; LSM5 and LSM7; and LSM7 and LSM4 (Figure 3C; see Supplemental Figure 4 online). These interactions parallel those proposed for LSM complexes from other eukaryotes (Beggs, 2005) and were observed simultaneously in both cytoplasm and nucleus (Figure 3C; see Supplemental Figure 4 online), consistent with the subcellular localization of the corresponding LSMs (see above). In addition, also according to the interactions assumed in other LSM complexes (Beggs, 2005), we did not detect interactions between LSM2 and LSM7, LSM4 and LSM6 (A or B), LSM6 (A or B) and LSM7, and LSM5 and LSM3 (A or B) (Figure 3C; see Supplemental Figure 4 online). All of these data indicate that *Arabidopsis* LSMs are organized in two heptameric ring complexes localized in the cytoplasm (LSM1-7) and the nucleus (LSM2-8).

In yeast and animals, cytoplasmic and nuclear LSM complexes are determined by the presence of LSM1 and LSM8 proteins, respectively (Tharun, 2009). The occurrence of a similar structural requirement in *Arabidopsis* complexes was examined by analyzing the subcellular distribution of LSM4, a protein marker of both *Arabidopsis* cytoplasmic and nuclear LSM complexes, in plants deficient in LSM1 and LSM8 proteins. As described above, in root cells from *Arabidopsis* seedlings containing the $LSM4_{pro}$-*LSM4-GFP* construct, the LSM4-GFP fusion protein was simultaneously detected in both cytoplasm and nucleus (Figure 3D). Interestingly, however, in mutant seedlings for *LSM1* and *LSM8* (*lsm1a lsm1b* and *lsm8*, respectively) bearing the same construct, the fusion protein preferentially localized in nuclei or cytoplasm, respectively (Figure 3D). These observations strongly support the notion that LSM1 and LSM8 proteins are essential for the formation of the cytoplasmic and nuclear LSM complexes, respectively, in *Arabidopsis*.

LSM1- and LSM8-Deficient *Arabidopsis* Mutants Display Severe Developmental Alterations

The results described above indicated that *Arabidopsis* LSMs are also organized in cytoplasmic and nuclear complexes determined by the presence of LSM1 and LSM8 and suggested similar functions as the complexes from yeast and animals. To test this assumption, we first searched for T-DNA insertion mutants in *LSM1* and *LSM8* genes. Plants containing single T-DNA insertions located in the fourth exon of *LSM1A* or in the second intron of *LSM1B* were identified (Figure 4A). *LSM1A* or *LSM1B* mRNAs were undetectable in plants homozygous for the insertions (Figure 4B), revealing that these new *LSM1A* and *LSM1B*

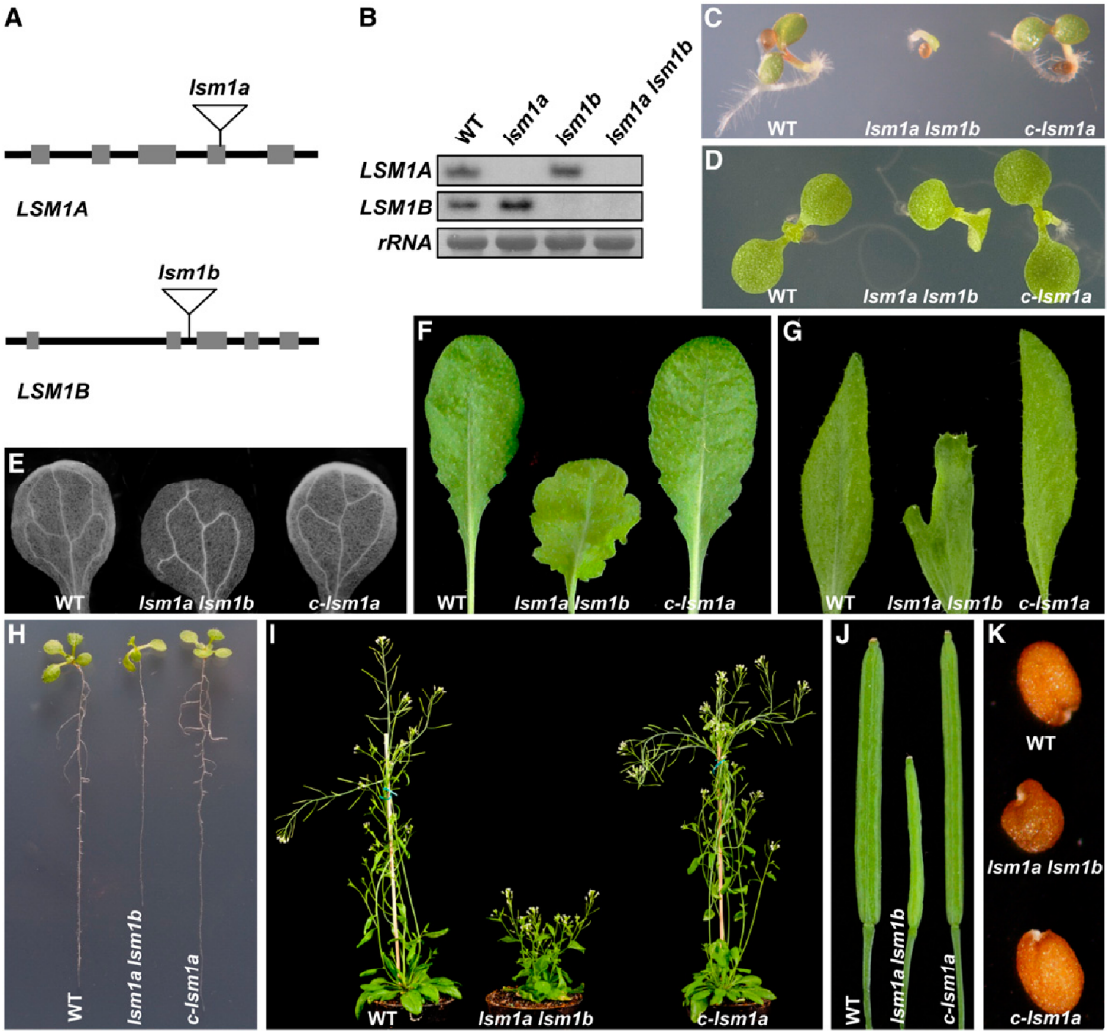

Figure 4. Phenotypic Analysis of *lsm1a lsm1b* Double Mutant.

(A) Schematic representation of *lsm1a* and *lsm1b* T-DNA insertions in *LSM1A* and *LSM1B* genes, respectively. Boxes symbolize exons.
(B) Expression analysis of *LSM1A* and *LSM1B* genes in 2-week-old wild-type (WT), *lsm1a*, *lsm1b*, and *lsm1a lsm1b Arabidopsis* plants by RNA hybridization using specific probes. *rRNA* levels are shown as a loading control.
(C) to **(K)** Morphological phenotypes of wild-type, *lsm1a lsm1b*, and *c-lsm1a* plants. Three-day-old seedlings **(C)**, 5-d-old seedlings **(D)**, cotyledon vein patterns **(E)**, rosette leaves **(F)**, cauline leaves **(G)**, 12-d-old seedlings **(H)**, 6-week-old plants **(I)**, siliques **(J)**, and seeds **(K)**.

alleles (*lsm1a* and *lsm1b*) were null or highly hypomorphic. Intriguingly, *lsm1a* and *lsm1b* plants did not present any obvious morphological or developmental abnormality, being indistinguishable from their corresponding wild-type plants, Nossen-0 (No-0) and Columbia-0 (Col-0) ecotypes, respectively (see Supplemental Figure 5 online).

Since LSM1A and LSM1B are 80% identical (see Supplemental Figure 1 online), they might be functionally redundant, which would explain the wild-type phenotypes exhibited by *lsm1a* and *lsm1b* single mutant plants. Therefore, we decided to obtain the *lsm1a lsm1b* double mutant that was subsequently backcrossed four times with Col-0 to have both mutations within this genetic background. As expected, *lsm1a lsm1b* plants did not accumulate *LSM1A* and *LSM1B* mRNAs (Figure 4B). Remarkably, in contrast with single mutants, the *lsm1a lsm1b* double mutant showed severe developmental alterations. Seed germination in *lsm1a lsm1b* was delayed compared with the wild type and disturbed, producing epinastic, chlorotic, and small cotyledons (Figures 4C and 4D; see Supplemental Figure 6A online). Cotyledonary veins were disorganized with disruptions, preventing the formation of closed loops as in wild-type veins (Figure 4E; see Supplemental Figure 6C online). *lsm1a lsm1b* rosette and cauline leaves were smaller than wild-type leaves, more serrated, and presented an abnormal venation phenotype and smaller petioles (Figures 4F and 4G; see Supplemental Figures 6D to 6F online). The root system was also altered in *lsm1a lsm1b* plants, with the root length and the number of secondary roots being reduced (Figure 4H; see

Supplemental Figures 6G and 6H online). On the other hand, the elongation of primary and secondary inflorescences ceased prematurely in the double mutant, altering plant architecture and given rise dwarf plants (Figure 4I). *lsm1a lsm1b* plants flowered earlier than wild-type plants under both long- and short-day photoperiods, though this phenotype was much more pronounced under noninductive photoperiodic conditions (see Supplemental Figures 6I and 6J online). Finally, mutant plants produced few siliques that were shorter and contained less seeds than those of wild-type plants (Figure 4J; see Supplemental Figures 6K and 6L online). Moreover, these seeds were small and frequently presented morphological alterations (Figure 4K). *lsm1a lsm1b* plants transformed with either *LSM1A*$_{pro}$*-LSM1A-GFP* (*c-lsm1a*) or *LSM1B*$_{pro}$*-LSM1B-GFP* (*c-lsm1b*) were rescued for all of the above phenotypes (Figures 4C to 4K; see Supplemental Figures 6 and 7 online), confirming that LSM1A and LSM1B are, in fact, functionally redundant and that the mutant phenotypes displayed by the double mutant were due to the absence of *LSM1A* and *LSM1B* expression.

In addition, two transgenic lines were identified that contained single T-DNA insertions located in the fifth exon of *LSM8* (Figure 5A). In homozygous plants for the insertions, *LSM8* mRNA was undetectable, indicating that these new *LSM8* alleles (*lsm8-1* and *lsm8-2*) were null or highly hypomorphic (Figure 5B). *lsm8-1* and *lsm8-2* mutants also exhibited developmental defects (Figure 5; see Supplemental Figure 6 online). Both of them showed the same phenotypes, but they were more pronounced in *lsm8-1*. Seeds from *lsm8* mutants germinated as wild-type seeds, although a significant percentage of mutant seedlings exhibited alterations in the shape and number of their cotyledons, and veins formed more closed loops in *lsm8* than in wild-type cotyledons (Figures 5C and 5D; see Supplemental Figures 6A to 6C online). *lsm8-1* and *lsm8-2* rosette leaves had short petioles and were smaller and flatter than wild-type leaves, but their vasculature and margins were normal (Figure 5E; see Supplemental Figures 6D to 6F online). Regarding the radicular system, the root length and the number of secondary roots were reduced in *lsm8* mutants compared with the wild type (Figure 5F; see Supplemental Figures 6G and 6H online). The length of primary and secondary inflorescences was not affected in the mutants (Figure 5G). Nonetheless, they flowered significantly earlier than wild-type plants under short-day photoperiods (see Supplemental Figures 6I and 6J online). Although the number of siliques produced in *lsm8* mutants was as in the wild type, they were shorter and contained fewer seeds that frequently aborted (Figures 5H and 5I; see Supplemental Figures 6K and 6L online). *lsm8-1* mutant plants transformed with the construct *LSM8*$_{pro}$*-LSM8-GFP* (*c-lsm8*) exhibited wild-type phenotypes (Figures 5C to 5I; see Supplemental Figure 6 online), confirming that their mutant phenotypes were due to the lack of *LSM8* expression. Altogether, these data provide direct evidence that LSM1 and LSM8 proteins are required to ensure correct developmental transitions in *Arabidopsis*, from germination to flowering, and also in seed formation.

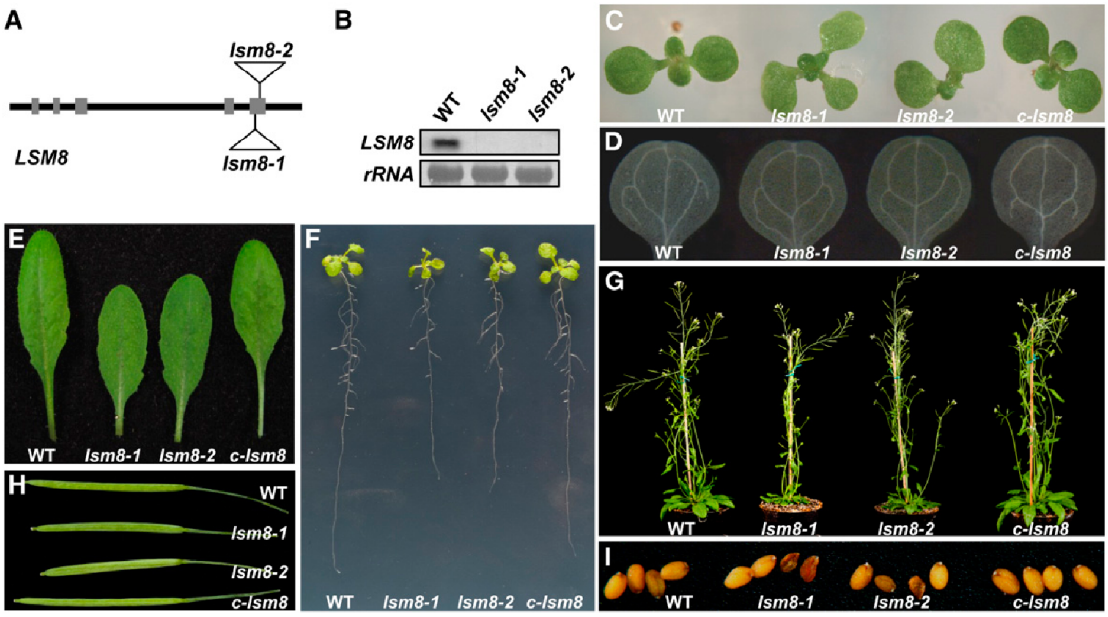

Figure 5. Phenotypic Analysis of *lsm8* Mutants.

(A) Schematic representation of *lsm8-1* and *lsm8-2* T-DNA insertions in the *LSM8* gene. Boxes symbolize exons.
(B) Expression analysis of *LSM8* in 2-week-old wild-type (WT), *lsm8-1*, and *lsm8-2 Arabidopsis* plants by RNA hybridization using a specific probe. *rRNA* levels are shown as a loading control.
(C) to **(I)** Morphological phenotypes of wild-type, *lsm8-1*, *lsm8-2*, and *c-lsm8* plants. Five-day-old seedlings **(C)**, cotyledon vein patterns **(D)**, rosette leaves **(E)**, 12-d-old seedlings **(F)**, 6-week-old plants **(G)**, siliques **(H)**, and seeds **(I)**.

Accumulation of Capped Transcripts and mRNA Stability Are Affected in *lsm1a lsm1b* Mutants

The possibility that the *Arabidopsis* LSM1-7 cytoplasmic complex functions in mRNA degradation, as described in yeast and animals (Bouveret et al., 2000; Tharun et al., 2000), was tested by analyzing the decay rates of several mRNAs that have been reported to be unstable transcripts, such as *EXPANSIN-LIKE1* (*EXPL1*), *ARABIDOPSIS ORTHOLOG OF HS1 PRO1-2* (*ATHSPRO2*), *JASMONATE-ZIM-DOMAIN PROTEIN6* (*JAZ6*), *ARABIDOPSIS NITRATE REDUCTASE2* (NIA2), *JAZ1*, and *RELATED TO ABI3/VP1 1* (RAV1) (Gutiérrez et al., 2002), in *lsm1a lsm1b* and wild-type plants. As a control, we also analyzed the turnover of *EUKARYOTIC TRASLATION INITIATION FACTOR 4A1* (*EIF4A1*) mRNA, which is considered a stable transcript (Gutiérrez et al., 2002). Decay rates were assayed by comparing relative levels of mRNAs following cordycepin-induced transcriptional arrest (Gutiérrez et al., 2002). Our results confirmed the instability of the former mRNAs and the stability of the latter in the wild type (Figure 6A). In *lsm1a lms1b*, however, the steady state levels of all unstable transcripts analyzed were higher than in wild-type plants and their rates of decay clearly reduced, their estimated half-lives (the time required for an mRNA to be reduced to half its initial value) being at least two times longer (Figures 6A and 6B). As expected, the steady state levels and the decay rate of *EIF4A1* RNA were similar in mutant and wild-type plants (Figures 6A and 6B). The analysis of the stability of *EXPL1*, *ATHSPRO2*, *JAZ6*, and *EIF4A1* transcripts in *c-lsm1a* and *c-lsm1b* plants confirmed that LSM1A and LSM1B are functionally redundant and demonstrated that the increased mRNA stability noticed in *lsm1a lms1b* was caused by the simultaneous absence of *LSM1A* and *LSM1B* expression (Figures 6C and 6D; see Supplemental Figures 8A and 8B online). We also examined the stability of *EXPL1*, *JAZ6*, and *EIF4A1* mRNAs in *lsm1a*, *lsm1b*, and *lsm8-1* single mutants. As presumed, all mRNAs showed similar turnover in cordycepin-treated wild-type and mutant plants (see Supplemental Figures 8C to 8H online), confirming again the functional redundancy of LSM1A and LSM1B and establishing that the *Arabidopsis* LSM2-8 nuclear complex does not play a role in cytoplasmic mRNA degradation.

We next assessed whether the reduction of mRNA decay observed in *lsm1a lms1b* could be due to a deficiency in its mRNA decapping capacity. For this, rapid amplification of cDNA ends (RACE)-PCR that allows detection of capped forms of specific mRNAs was used. PCR experiments with low and high number of cycles were performed. In both cases, we found that *EXPL1*, *ATHSPRO2*, and *JAZ6* mRNAs accumulated in their capped form in the *lsm1a lsm1b* mutant compared with the wild type (Figure 6E). These effects were corrected by expression of *LSM1A* and *LSM1B* transgenes in *c-lsm1a* and *c-lsm1b* plants, respectively (Figure 6E). The capped forms of mRNAs corresponding to the above genes were found not to be changed in *lsm1a*, *lsm1b*, and *lsm8-1* mutants (see Supplemental Figure 8I online). These results indicated that the *Arabidopsis* LSM1-7 complex operates in cytoplasmic mRNA degradation by promoting decapping.

Loss of LSM8 Influences U6 snRNA Stability and Results in pre-mRNA Splicing Defects

In yeast and animals, the LSM2-8 nuclear complex acts in pre-mRNA splicing by stabilizing the spliceosomal U6 snRNA (Beggs, 2005). To determine whether the *Arabidopsis* LSM nuclear complex has a similar function, we first analyzed the effects of LSM8 on pre-mRNA splicing at the genome-wide level using tiling arrays (Affymetrix *Arabidopsis* Tiling 1.0R) and total RNAs from wild-type and *lsm8-1* mutant plants. Two-week-old plants were selected for these experiments as they represent an intermediate stage of development. We searched for introns with significantly higher hybridization signals in mutant than in wild-type plants. Thus, we identified 469 introns, belonging to 453 genes, with increased hybridization signals in *lsm8-1* (see Supplemental Data Set 2A online). The increased hybridization signals detected in *lsm8-1* should reflect intron retention since hybridization signals in other introns and exons of the genes did not differ between wild-type and *lsm8-1* plants. These results were validated by RT-PCR for a subset of genes appertaining to different ontology categories, including protein metabolism (*AT1G17960*), intracellular transport (*AT3G59390*), developmental processes (*ARABIDOPSIS THALIANA PROTEIN ARGININE METHYLTRASFERASE 4A*, *PRMT4A*) or signal transduction (*CASEIN KINASE-LIKE5*, *CKL5* and *PROTEIN KINASE AME3*, *AME3*), in both *lsm8-1* and *lsm8-2* mutants (Figure 7A; see Supplemental Figure 9 online). However, the intron retention events in these genes were not detected in *c-lsm8* and *lsm1a lsm1b* plants (Figure 7A), confirming that the splicing defects unveiled in *lsm8* mutants were specifically due to the loss of *LSM8* function and that the *Arabidopsis* LSM1-7 cytoplasmic complex is not involved in pre-mRNA splicing. As a control, tiling array data were also validated by analyzing the retention of an intron of *ACTIN-RELATED PROTEIN4*, *ARP4*, a gene that did not display any intron retention event in the array, in *lsm8-1* and *lsm8-2* mutants. As expected, the intron was not retained in these plants (Figure 7A).

Next, we investigated the possible role of the *Arabidopsis* LSM nuclear complex in U6 snRNA stability by assessing the levels of this snRNA in cordycepin-treated *lsm8* mutant and wild-type plants. Results revealed that the steady state levels of U6 snRNA were lower in mutants than in wild-type plants and that after cordycepin treatment these levels were maintained in wild-type plants but decreased rapidly in *lsm8-1* and *lsm8-2* mutants (Figure 7B). Therefore, the stability of U6 snRNA is dependent on the presence of LSM8 and, consequently, on the LSM2-8 nuclear complex. The effect of LSM8 on U6 snRNA stability seems to be highly specific since the levels of U3 small nucleolar RNA (snoRNA), which is transcribed by RNA polymerase III like the U6 snRNA, and U4 snRNA, which is synthesized by RNA polymerase II, did not decrease in cordycepin-treated *lsm8* mutants (Figure 7B). As expected, *c-lsm8* and *lsm1a lsm1b* plants showed similar levels of U6 snRNA, U3 snoRNA, and U4 snRNA as the wild type before and after cordycepin treatment (Figure 7C; see Supplemental Figure 10 online). Therefore, it was concluded that the *Arabidopsis* LSM2-8 nuclear complex is essential for accurate splicing of selected mRNAs through the stabilization of the spliceosomal U6 snRNA.

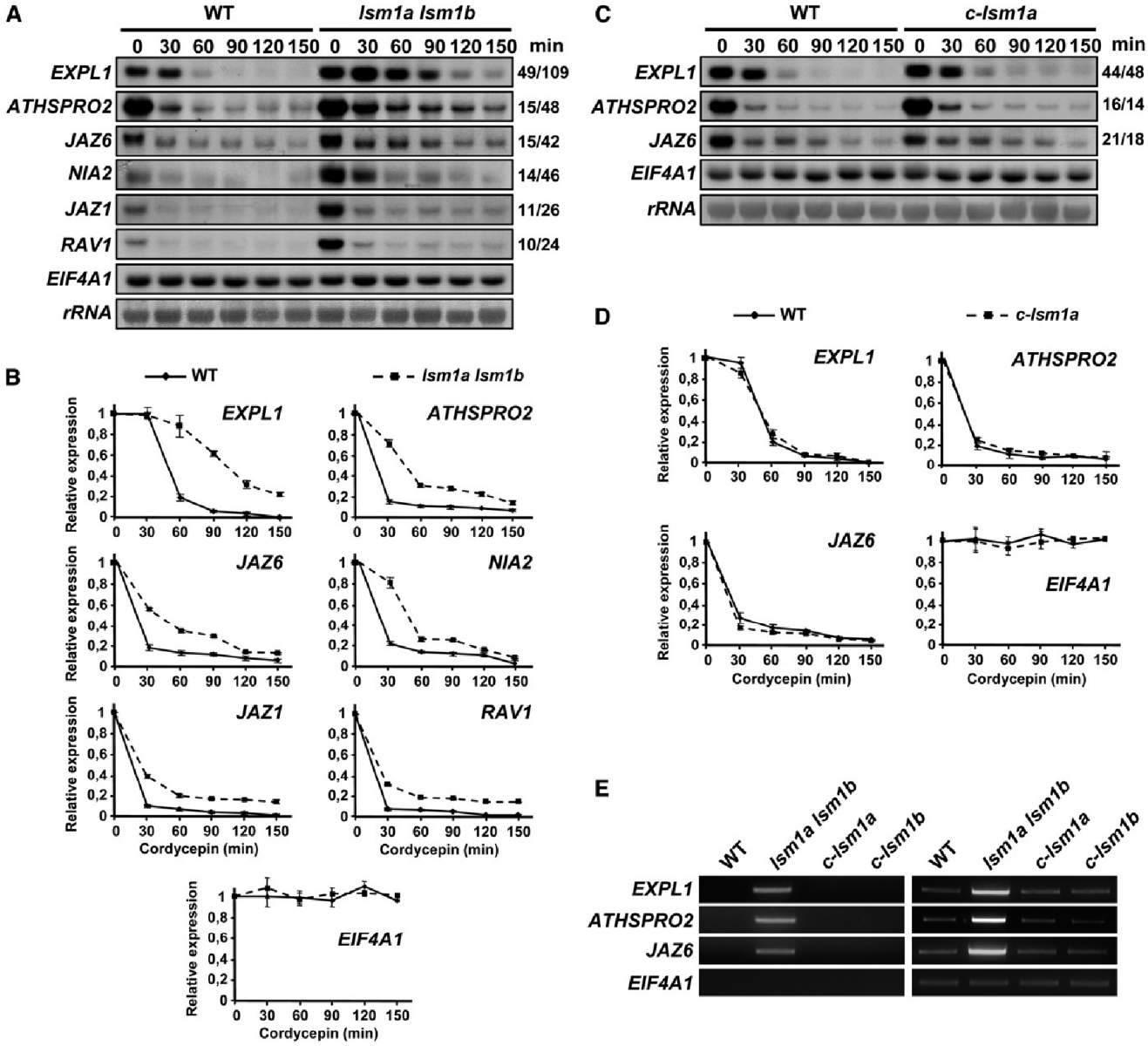

Figure 6. mRNA Stability and Accumulation of Capped Transcripts in the *lsm1a lsm1b* Double Mutant.

(A) to **(D)** Transcript accumulation in *lsm1a lsm1b* and *c-lsm1a* plants. Levels of several transcripts in 6-d-old *Arabidopsis* seedlings of the wild type (WT) and *lsm1a lsm1b* (**[A]** and **[B]**) and of the wild type and *c-lsm1a* (**[C]** and **[D]**) at different minutes after cordycepin treatment.

(A) and **(C)** RNA hybridizations using specific probes. *rRNA* levels were used as a loading control. The estimated half-life (min) of mRNAs is shown to the right of each panel (wild type/analyzed genotype).

(B) and **(D)** Normalized quantification of the hybridization bands corresponding to genes of **(A)** (shown in **[B]**) and **(C)** (shown in **[D]**).

(E) Accumulation of capped transcripts corresponding to different genes in 6-d-old wild-type, *lsm1a lsm1b*, *c-lsm1a*, and *c-lsm1b Arabidopsis* seedlings by RACE-PCR. RACE-PCR products obtained using a low (left panel) and high (right panel) number of cycles are shown. The products of *EIF4A1*, also derived from RACE-PCR, were used as a loading control.

Arabidopsis Mutants Deficient in LSM1 or LSM8 Proteins Exhibit Altered Development-Related Gene Expression

In an attempt to understand the function of LSM complexes in *Arabidopsis* development, we studied the global impact of *lsm1* and *lsm8* mutations on gene expression. The comparison of mRNA profiles from *lsm1a lsm1b* and the wild type was performed using Agilent *Arabidopsis* Oligo Microarrays v4 and total RNAs extracted from 2-week-old plants. Transcript levels of 358 genes were found to be higher, by at least twofold, in *lsm1a*

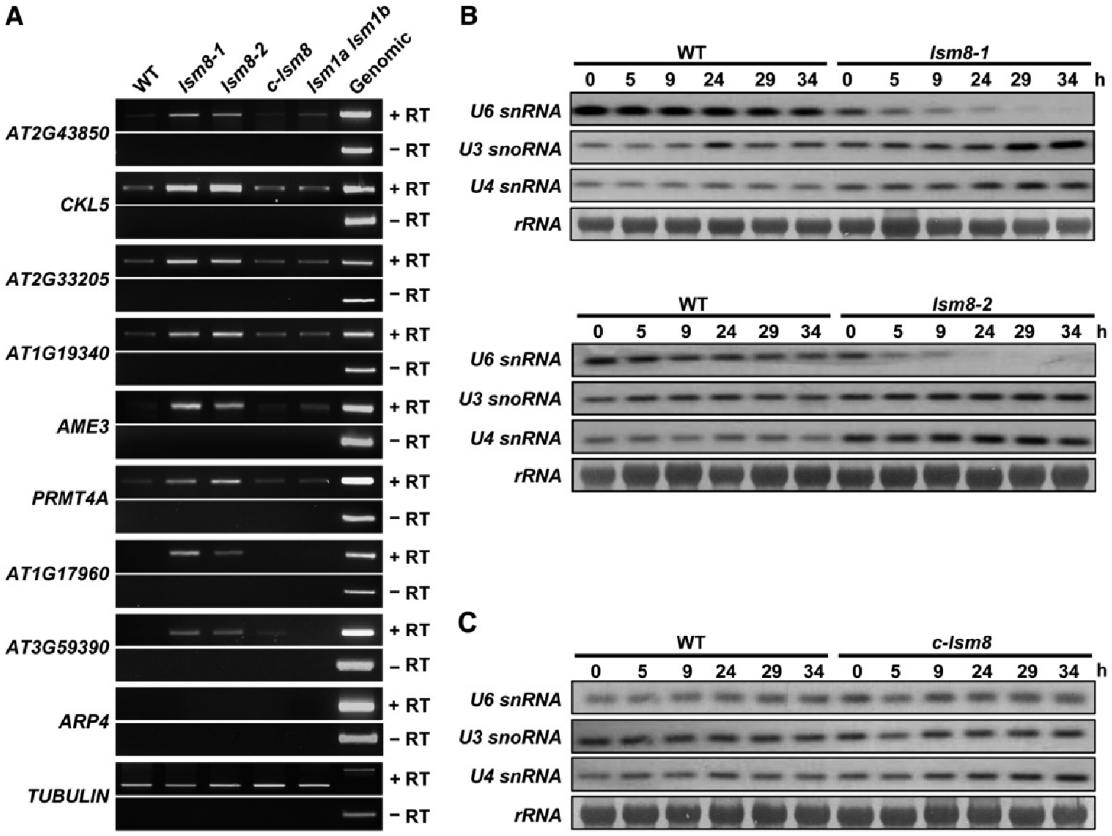

Figure 7. Intron Retention and U6 snRNA Stability in *lsm8* Mutants.

(A) Validation of intron retention events in different genes identified by tiling arrays in the *lsm8-1* mutant. RT-PCR was performed with total RNA from 2-week-old wild-type (WT), *lsm8-1*, *lsm8-2*, *c-lsm8*, and *lsm1a lsm1b Arabidopsis* plants and specific pairs of primers for each gene. In all cases, one primer was situated inside the retained intron and the other in an adjacent exon. Genomic DNA (Genomic) was used as a control. +RT indicates reactions with reverse transcriptase (RT). Control reactions without RT (−RT) were also performed. *TUBULIN* expression is shown as a loading control. **(B)** and **(C)** Stability of U6 snRNA in *lsm8-1*, *lsm8-2*, and *c-lsm8* plants. Levels of U6 snRNA, U3 snoRNA, and U4 snRNA in 6-d-old *Arabidopsis* seedlings of the wild type, *lsm8-1*, and *lsm8-2* **(B)** and of the wild type and c-*lsm8* **(C)** at different hours after cordycepin treatment, as shown by RNA hybridization using specific probes. *rRNA* levels were used as a loading control.

lsm1b than in the wild type (see Supplemental Data Set 2B online). On the other hand, transcripts corresponding to 316 genes were reduced by more than twofold in *lsm1a lsm1b* compared with the wild type (see Supplemental Data Set 2B online). Gene ontology analysis of deregulated genes in the double mutant unveiled that 72 of them were implicated in developmental processes, including seed germination, root and leaf development, inflorescence development, flowering, and embryogenesis (see Supplemental Data Set 2B online), which is consistent with its severe mutant phenotype (Figures 4C to 4K). The microarray data were validated, confirming the altered expression of several overexpressed and underexpressed genes related to different developmental processes in *lsm1a lsm1b* plants by quantitative RT-PCR (Figures 8A and 8B). On the other hand, *c-lsm1a* and *c-lsm1b* plants exhibited wild-type expression patterns for all validated genes (Figures 8A and 8B), demonstrating that the LSM1-7 cytoplasmic complex is required for the accurate expression of development-related genes in *Arabidopsis*.

Since the *Arabidopsis* LSM cytoplasmic complex functions in mRNA degradation by promoting decapping (see above), the high levels of some development-related mRNAs detected in the absence of LSM1 proteins might be due to a selective stabilization of the corresponding transcripts as a result of the retention of their 5′ cap. This possibility was first examined by measuring the degradation rates of five development-related mRNAs (*YELLOW-LEAF-SPECIFIC GENE9* [*YLS9*], *UDP-GLUCOSYL TRANSFERASE 87A2* [*UGT87A2*], *ARABIDOPSIS THALIANA EXPANSIN-LIKE14* [*ATEXP14*], *MATERNAL EFFECT EMBRYO ARREST14* [*MEE14*], and *ARABIDOPSIS THALIANA HOMEOBOX12* [*ATHB12*]), whose levels were elevated in the *lsm1a lsm1b* double mutant, in cordycepin-treated wild-type and *lsm1a lsm1b* plants. Interestingly, the decay of all transcripts, except *ATHB12*, was significantly slower in the mutant than in wild-type plants (Figure 8C). In addition, all transcripts, but not *ATHB12*, retained their 5′ cap in *lsm1a lsm1b* (Figure 8D), providing evidence that, in fact, the *Arabidopsis* LSM1-7 cytoplasmic complex is essential for correct developmental gene expression by regulating the decapping and,

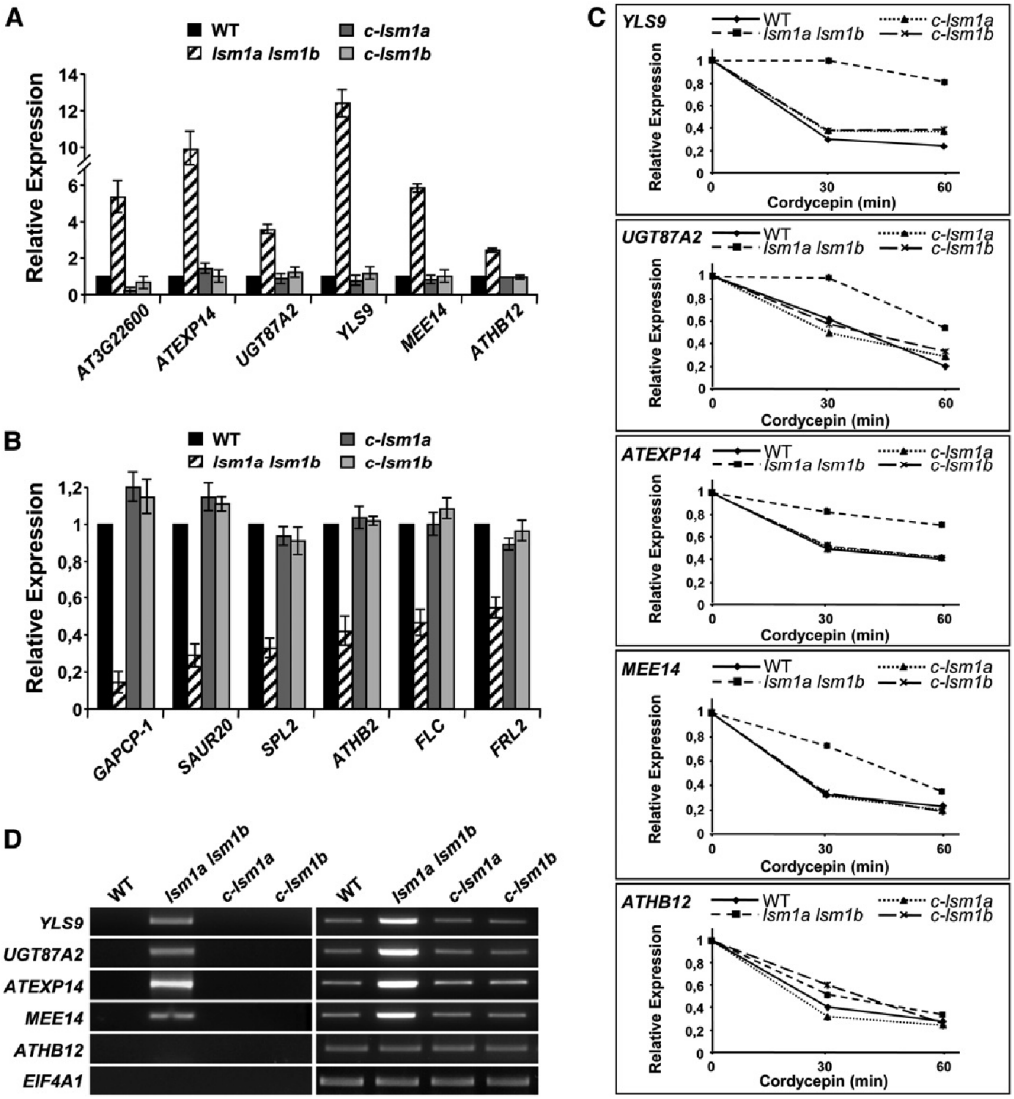

Figure 8. Accumulation of Development-Related Transcripts in the *lsm1a lsm1b* Double Mutant.

(A) and **(B)** Expression levels of different developmental genes detected in the microarray with altered expression in *lsm1a lsm1b*. The relative levels of 12 RNAs that in the microarray were increased **(A)** or decreased **(B)** are shown. Real-time RT-PCR analyses were performed with total RNA from 2-week-old wild-type (WT), *lsm1a lsm1b*, *c-lsm1a*, and *c-lsm1b Arabidopsis* plants and specific pairs of primers for each gene.

(C) Accumulation of transcripts corresponding to several developmental genes detected in the microarray with increased expression in *lsm1a lsm1b*. In all cases, the relative transcript levels were determined by real-time RT-PCR analysis, as described above, in wild-type, *lsm1a lsm1b*, *c-lsm1a*, and *c-lsm1b Arabidopsis* plants at different minutes after cordycepin treatment. Values are relative to the control values obtained for each genotype.

(D) Accumulation of capped transcripts corresponding to genes analyzed in **(C)** in 2-week-old wild-type, *lsm1a lsm1b*, *c-lsm1a*, and *c-lsm1b Arabidopsis* plants by RACE-PCR. RACE-PCR products obtained using a low (left panel) and high (right panel) number of cycles are shown. The products of *EIF4A1*, also derived from RACE-PCR, were used as a loading control.

therefore, the stabilization of specific development-related transcripts. Accordingly, the degradation rates and cap levels of *YLS9*, *UGT87A2*, *ATEXP14*, *MEE14*, and *ATHB12* transcripts in *c-lsm1a* and *c-lsm1b* plants were as in the wild type (Figures 8C and 8D).

The effect of *lsm8* mutations on gene expression at a genome-wide level was determined analyzing the above-mentioned tiling arrays, which, in addition to allowing splicing analysis, constitute a robust platform for detection of transcriptional activity (Laubinger et al., 2008). Compared with the wild type, 65 and 193 annotated genes were found to be at least twofold up- and downregulated, respectively, in the *lsm8-1* mutant (see Supplemental Data Set 2C online). Gene ontology categorization of these deregulated genes revealed that a considerable number (17 upregulated and 50 downregulated) were related to developmental processes throughout the *Arabidopsis* life cycle (see Supplemental Data Set 2C online), which could explain the mutant phenotypes

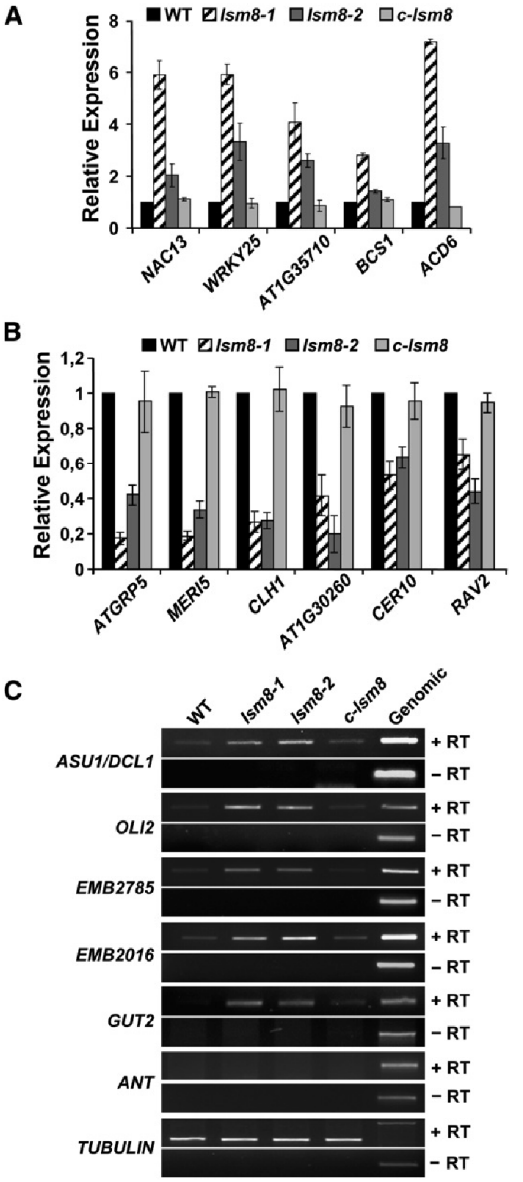

Figure 9. Intron Retention in Developmental Genes in *lsm8* Mutants.

(A) and **(B)** Expression levels of different development-related genes detected in the tiling array with altered expression in *lsm8* mutants. The relative levels of 11 RNAs that were increased **(A)** or decreased **(B)** in the array are shown. Real-time RT-PCR analyses were performed with total RNA from 2-week-old wild-type (WT), *lsm8-1*, *lsm8-2*, and *c-lsm8 Arabidopsis* plants and specific pairs of primers for each gene.

(C) Validation of intron retention events in some developmental genes identified by tiling arrays in *lsm8-1*. RT-PCR was performed with total RNA from 2-week-old wild-type, *lsm8-1*, *lsm8-2*, and *c-lsm8 Arabidopsis* plants and specific pairs of primers for each gene. In all cases, one primer was situated inside the retained intron and the other in an adjacent exon. Genomic DNA (Genomic) was used as a control. +RT indicates reactions with reverse transcriptase (RT). Control reactions without RT (−RT) were also performed. *TUBULIN* expression is shown as a loading control.

exhibited by *lsm8* mutants (Figures 5C to 5I). Microarray results were validated by assaying the expression of a group of deregulated genes implicated in different developmental processes in wild-type, *lsm8-1*, *lsm8-2*, and *c-lsm8* plants by quantitative RT-PCR (Figures 9A and 9B). These data demonstrated that the *Arabidopsis* LSM2-8 nuclear complex is also crucial for appropriate development-related gene expression.

Considering that the LSM2-8 nuclear complex regulates development-related gene expression and functions in pre-mRNA splicing (see above), it was presumed that a number of genes involved in development might display splicing defects. Remarkably, 65 out of the 453 genes that showed intron retention events were found to be related to different developmental processes (see Supplemental Data Set 2A online). The inefficient splicing of some of these genes, including *ABNORMAL SUSPENSOR1/DICER-LIKE1* (*ASU1/DCL1*), *OLIGOCELLULA2* (*OLI2*), *EMBRYO DEFECTIVE 2785* (*EMB2785*), *EMBRYO DEFECTIVE 2016* (*EMB2016*), and *GLUCURONOXYLAN GLUCURONOSYLTRANSFERASE* (*GUT2*), in the absence of LSM8 was confirmed by RT-PCR analysis with appropriate primers (Figure 9C). As expected, the splicing of other developmental-related genes, such as *AINTEGUMENTA* (ANT), was not affected (Figure 9C). These findings indicate that the *Arabidopsis* LSM2-8 nuclear complex ensures the accurate splicing of specific development-related mRNAs, allowing correct developmental gene expression.

DISCUSSION

Although LSM-related proteins have been found in the genomes of different plant species (Proost et al., 2009; Goodstein et al., 2012), they have not yet been biochemically characterized and their function in RNA metabolism remained to be established. In this study, we used genetic, molecular, cell biology, and biochemical studies to demonstrate that *Arabidopsis* LSMs are organized in two heptameric complexes. More importantly, our results reveal that these complexes are essential for normal *Arabidopsis* development, and this role seems to be performed by controlling the proper turnover and splicing of selected developmental-related mRNAs that, in turn, ensures the appropriate gene expression during plant development.

Subcellular localization and BiFC experiments strongly support the idea that *Arabidopsis* LSM proteins assemble into two heteroheptameric complexes that differ by a single subunit, LSM1A/B or LSM8, and localize in cytoplasm (LSM1-7) and nucleus (LSM2-8). First, in *Arabidopsis*, as in other eukaryotes (Beggs, 2005), LSM1 proteins (LSM1A and LSM1B) specifically accumulate in the cytoplasm, while LSM8 has a nuclear localization and the rest of LSM proteins are simultaneously localized in cytoplasm and nucleus. Second, *Arabidopsis* LSM proteins do not interact promiscuously with each other. Instead, each LSM specifically interacts with two other LSM proteins following the same pattern of interaction as in the yeast and human heptameric complexes (Beggs, 2005). Consistent with their different subcellular localization and with the assumption that they define the two *Arabidopsis* LSM complexes, LSM1 and LSM8 proteins do not interact with each other. Moreover, while all

interactions involving LSM1 proteins take place in the cytoplasm, those involving LSM8 occur in the nucleus and those involving LSM2-7 proteins occur simultaneously in both subcellular compartments. Third, LSM1 and LSM8 proteins are required for the formation of the *Arabidopsis* cytoplasmic and nuclear LSM complexes, respectively.

Our genetic and molecular analyses allowed us to establish the function of *Arabidopsis* LSM complexes. In *Arabidopsis* plants deficient in LSM1 proteins, several transcripts accumulate in their capped forms and show a reduced degradation rate with the corresponding increase in their half-lives, indicating that the *Arabidopsis* LSM1-7 complex function in the 5′ to 3′ pathway of mRNA decay as an activator of decapping. As expected from their high amino acid identity (80%), LSM1A and LSM1B are functionally redundant. *lsm1a* and *lsm1b* single null mutants are not perturbed in mRNA decapping and decay, and LSM1A and LSM1B, individually, are able to complement the alterations in mRNA decapping and decay displayed by the *lsm1a lsm1b* double mutant. On the other hand, *Arabidopsis* plants lacking LSM8 are affected in the stability of the spliceosomal U6 snRNA which, accordingly, results in pre-mRNA splicing defects. Compared with the wild type, at least 469 intron retention events distributed among 453 genes were detected in the *lsm8-1* mutant under our experimental conditions, demonstrating that the LSM2-8 complex regulates genome-wide pre-mRNA splicing. Although intron retention constitutes the most frequent splicing defect in plant genes (Syed et al., 2012), it is probable that other mRNA splicing defects, including exon skipping, alternative 5′ splicing, and alternative 3′ splicing, also occur in the absence of LSM8. Unfortunately, however, the detection of these defects is unreliable when using tiling arrays to analyze pre-mRNA splicing at global level (Ner-Gaon and Fluhr, 2006). The existence of splicing defects has also been reported in some genes of plants harboring a postembryonic lethal mutation in *LSM4* (Zhang et al., 2011). Nonetheless, only one of these genes (*AT1G28060*) has been found in our tiling analysis of the *lsm8-1* mutant, in all likelihood because of the plants used being at different developmental stages and the different methods of analysis being used. Furthermore, only a few genes were analyzed for splicing defects in the *lsm4* mutant (Zhang et al., 2011). The fact that not all mRNAs exhibit reduced degradation rates in *lsm1a lsm1b* plants nor splicing defects in *lsm8* mutants indicates that the cytoplasmic and nuclear LSM complexes from *Arabidopsis*, as described for other components of the *Arabidopsis* machineries involved in mRNA degradation and processing (Xu et al., 2006; Goeres et al., 2007; Xu and Chua, 2009; Kim et al., 2010; Rymarquis et al., 2011), act on selected targets. How selected mRNAs are targeted to these complexes remains largely unknown. According to their relevant function in mRNA decapping and degradation, the lack of LSM1 proteins has a deep impact on *Arabidopsis* gene expression, the levels of more than 600 transcripts being significantly altered, 358 increased and 316 reduced, in *lsm1a lsm1b* plants. Similarly, the expression of at least 250 genes is significantly affected in null mutants for *LSM8*. Consistent with the implication of *Arabidopsis* LSM8 in pre-mRNA splicing, in this case the number of downregulated (193) genes is much higher than that of upregulated (65) ones. These data indicate that, like other factors involved in the *Arabidopsis* decapping 5′ to 3′ decay pathway, including DCP2, DCP5, and XRN4, or in pre-mRNA splicing, such as STABILIZED1 (STA1) (Lee et al., 2006; Xu et al., 2006; Goeres et al., 2007; Xu and Chua, 2009; Rymarquis et al., 2011), the *Arabidopsis* LSM1-7 and LSM2-8 complexes also play a major role in maintaining appropriate levels of gene expression. Interestingly, however, the result of the absence of these factors on *Arabidopsis* gene expression seems to be highly specific.

In eukaryotic cells, P-bodies appear as cytoplasmic foci containing RNP complexes associated with translational repression, mRNA storage, and cytoplasmic mRNA decay pathways (Xu and Chua, 2011). Under conditions promoting high levels of mRNA turnover, such as osmotic, hypoxic, or heat stress conditions, P-bodies increase in number and size, becoming more apparent (Teixeira et al., 2005; Weber et al., 2008). Nevertheless, it is not yet clear how P-bodies are formed and what their function is in eukaryotic cells. Human LSM4 localizes in P-bodies and loses this localization when mutations are introduced in residues involved in interacting with other LSM proteins (Ingelfinger et al., 2002). In yeast, LSM2 and LSM7 fail to localize to P-bodies in LSM1-deficient cells (Tharun et al., 2005), and it has been shown that LSM4 plays a role in the localization of the LSM1-7 complex in P-bodies and in P-body assembly (Decker et al., 2007; Reijns et al., 2008). Our findings demonstrate that the *Arabidopsis* LSM1-7 complex not only accumulates in P-bodies, which is consistent with its function in cytoplasmic mRNA decapping and decay but is also essential for their formation. As expected from its specific nuclear localization, LSM8 does not localize in P-bodies. The implication of *Arabidopsis* LSM2-LSM7 proteins in P-body formation is difficult to assess due to the absence of viable *lsm2-lsm7* null mutants (see below). To date, only few proteins have been related with P-bodies in plants, including DCP5, an *Arabidopsis* protein indirectly implicated in regulating mRNA decapping that has a function in P-body formation (Xu and Chua, 2009), and DCP1, DCP2, and VCS, three proteins that constitute a decapping complex and colocalize with P-bodies in *Arabidopsis* (Xu et al., 2006; Goeres et al., 2007). In addition, *Arabidopsis* proteins XRN4, *ARABIDOPSIS THALIANA* TANDEM ZINC FINGER PROTEIN1, and POLYPYRIMIDINE TRACT BINDING PROTEINS have also been found in plant P-bodies (Weber et al., 2008; Pomeranz et al., 2010; Stauffer et al., 2010). The identification of additional P-body components will certainly help to understand how they are formed and what their function is in plant cells.

It has been described that the proteins involved in mRNA decapping, DCP1, DCP2, VCS, and DCP5, as well as the splicing factors STA1, U11/U112-31K, and ERECTA MRNA UNDER-EXPRESSED, play an essential role in *Arabidopsis* development, their absence being lethal (Lee et al., 2006; Xu et al., 2006; Goeres et al., 2007; Xu and Chua, 2009; Furumizu et al., 2010; Kim et al., 2010). *Arabidopsis* plants deficient in LSM1 and LSM8 proteins also display quite severe development alterations, but they are viable. *lsm1a lsm1b* and *lsm8* mutants are affected in both vegetative and reproductive developmental traits, indicating that cytoplasmic and nuclear LSM complexes are required for the normal development of *Arabidopsis* throughout the different phases of its life cycle. Nevertheless, consistent with

the different function of the two LSM complexes, the phenotypes exhibited by *lsm1a lsm1b* and *lsm8* mutants are different. Recently, T-DNA insertional mutations for *LSM4* and *LSM7* have been described to show postembryonic and embryonic lethality, respectively (Zhang et al., 2011; http://www.seedgenes.org/). We have observed the same lethal phenotype not only for the *lsm4* and *lsm7* null mutations but also for the *lsm3a lsm3b* and *lsm6a lsm6b* doubles, with the corresponding single mutants exhibiting wild-type phenotypes, as well as for the *lsm1a lsm1b lsm8* triple mutations (C. Perea-Resa, T. Hernández-Verdeja, and J. Salinas, unpublished data). Moreover, we have not found any insertion abolishing the expression of *LSM2* and *LSM5* genes in the available T-DNA collections, which suggests that, probably, *lsm2* and *lsm5* null mutations are also lethal. However, weak mutant alleles for these genes do not appear to be lethal. In fact, a point mutation in *LSM5* (*sad1*) that causes the conversion of a Glu residue to a Lys makes mutant plants much smaller than the wild type but does not result in lethality (Xiong et al., 2001). Altogether, these data indicate that the presence of at least one LSM complex is essential in *Arabidopsis*. In yeast, it has been proposed that LSM2-7 proteins might associate, in the apparent absence of LSM1 or LSM8, with other proteins, including related SM proteins, to form complexes that would remain at least partially active (Beggs, 2005). We cannot exclude that this could be the case in *lsm1a lsm1b* and *lsm8* mutants. Further studies are required to understand how *Arabidopsis* can develop and reproduce with just one LSM complex.

The results presented in this work demonstrate that posttranscriptional regulation has an important role in controlling gene expression related to plant development. In fact, we show that several selected genes involved in both vegetative and reproductive development are targets of the *Arabidopsis* LSM complexes. Thus, the LSM1-7 cytoplasmic complex ensures the precise half-life of the transcripts corresponding to its targets, for instance, *UGT87A2* (floral transition), *MEE14* (embryo development), or *YLS9* (leaf development), and, consequently, their adequate temporal expression patterns. The LSM2-8 nuclear complex, in turn, guarantees the correct splicing of its targets, such as *ASU1*/*DCL1* (flower development), *OLI2* (leaf development), or *EMB2785* (embryo development) and, therefore, the accurate translation of the corresponding transcripts. Furthermore, we also show that, consistent with their role in turnover and splicing of development-related mRNAs, the *Arabidopsis* LSM complexes regulate the expression levels of many genes that are implicated in different developmental processes, including seed germination, root development, leaf development, floral transition, flower development, and embryogenesis. In particular, the expression levels of 72 and 67 specific genes involved in development were found to be altered in *lsm1a lsm1b* and *lsm8-1* mutants, respectively. However, it is obvious that these numbers should be considerably higher taking into account that only plants from one developmental stage (2 weeks old) were analyzed by microarray experiments. We propose that the cumulative defects in gene expression are responsible for the abnormal developmental phenotypes observed in these plants.

In conclusion, the findings presented here reveal the organization and function of *Arabidopsis* LSM proteins and demonstrate that these proteins are crucial for plant growth and development. Understanding the molecular mechanisms that regulate the function of LSMs and confer their target specificity constitutes an interesting challenge for the future.

METHODS

Plant Material, Constructs, and Growth Conditions

Arabidopsis thaliana Col-0 ecotype and mutants *lsm8-1* (Salk-025064) and *lsm8-2* (Salk-048010) were obtained from the Nottingham Arabidopsis Stock Center. Mutant *lsm1b* is a Gabi-kat line from Max Plank Institute (GK 391E05). *Arabidopsis* No-0 ecotype and mutant *lsm1a* (12-2253-1) were obtained from Riken Institute. *lsm1a* is a Ds transposon insertion line in the No-0 background. *lsm1a* and *lsm1b* single mutants were crossed to generate a *lsm1a lsm1b* double mutant that was subsequently backcrossed four times with Col-0 to have both mutations within this genetic background. Transgenic Col-0 plants containing the *35S-GFP-DCP2* and *35S-GFP-VCS* constructs (Goeres et al., 2007) were kindly provided by Leslie Sieburth (University of Utah, Salt Lake City, UT). These plants were crossed with *lsm1a lsm1b* to obtain double mutants with the *35S-GFP-DCP2* and *35S-GFP-VCS* constructs in homozygosis. All mutant and transgenic lines were genotyped using the primers listed in Supplemental Data Set 2D online.

To obtain the *LSMs*$_{pro}$*-GUS* fusions, at least 1-kb promoter fragment from each of the 11 *Arabidopsis LSM* genes was cloned into the pBI101 binary vector (Clontech). For the *LSMs*$_{pro}$*-LSM-GFP* fusions, genomic regions containing the *LSM1A*, *LSM1B*, *LSM3A*, *LSM3B*, *LSM4*, and *LSM8* genes, including at least 1 kb of the corresponding promoters, were cloned into the pGWB4 gateway binary vector (Nakagawa et al., 2007). All fusions were verified by sequencing and introduced in Col-0 via *Agrobacterium tumefaciens* C58C1 using the floral dip method (Clough and Bent, 1998). Fusions *LSM1A*$_{pro}$*-LSM1A-GFP*, *LSM1B*$_{pro}$*-LSM1B-GFP*, and *LSM8*$_{pro}$*-LSM8-GFP* were also introduced in *lsm1a lsm1b* and *lsm8-1* mutants. Similarly, the *LSM4*$_{pro}$*-LSM4-GFP* fusion was used to transform *lsm1a lsm1b* and *lsm8-1* mutants. Finally, the fusion *35S-RFP-DCP1* (Weber et al., 2008), kindly provided by Markus Fauth (Johann Wolfgang Goethe-University Frankfurt, Germany), was introduced in transgenic lines containing *LSM1A*$_{pro}$*-LSM1A-GFP* or *LSM1B*$_{pro}$*-LSM1B-GFP*. All transgenic lines were genetically determined to have the constructs integrated at a single locus in homozygosis. For BiFC assays, full-length cDNAs corresponding to the 11 *LSM* genes were amplified with appropriate primers (see Supplemental Data Set 2D online) to incorporate convenient restriction sites at their 5′ and 3′ ends. Fragments were cloned into the pSPYNE-35S and pSPYCE-35S binary vectors (Walter et al., 2004), kindly provided by Jörg Kudla (Westfälische Wilhelms-Universität Münster, Germany), sequenced, and introduced in *Agrobacterium* C58C1 for subsequent agroinfiltration. Agroinfiltration was performed in leaves from 3-week-old plants of *Nicotiana benthamiana* grown at 25°C, essentially as described (English et al., 1997), without using a silencing suppressor. The expression of fusion proteins was subsequently assayed 3 d after agroinfiltration.

Plants were grown at 20°C under long-day photoperiods (16 h of cool-white fluorescent light, photon flux of 90 μmol m^{-2} s^{-1}) in pots containing a mixture of organic substrate and vermiculite (3:1 [v/v]) or in Petri dishes containing Murashige and Skoog medium supplemented with 1% Suc and solidified with 0.8% (w/v) agar. Plants used to estimate flowering time in short-day conditions were grown under an 8-h light regime.

Gene Expression Analysis

Total RNA was extracted using the Purezol reagent (Bio-Rad) according to the manufacturer's protocol. RNA samples were treated with DNase I (Roche) and quantified with a Nanodrop spectrophotometer (Thermo

Scientific). RNA blot hybridizations were performed according to standard procedures. Specific probes were obtained by PCR with the primers described in Supplemental Data Set 2D online and labeled with [α-^{32}P] dCTP using the Megaprime DNA labeling systems kit (GE Healthcare). Equal RNA loading in the experiments was monitored by *rRNA* staining. RNA samples for each experiment were analyzed in at least three independent blots, and each experiment was repeated at least twice. For real-time RT-PCRs, cDNAs were prepared with the iScript cDNA synthesis kit (Bio-Rad) and then amplified using the Bio-Rad iQ2 thermal cycler, the SsoFast EvaGreen Supermix (Bio-Rad), and gene-specific primers (see Supplemental Data Set 2D online). The relative expression values were determined using the *AT4G24610* gene as a reference (Czechowski et al., 2005). All reactions were realized in triplicate employing three independent RNA samples.

Determination of GUS Activity

GUS activity in *Arabidopsis* transgenic plants containing the fusion *LSMs$_{pro}$-GUS* was detected and measured as previously described (Medina et al., 2001).

Microscopy Analysis

Subcellular localization of fusion proteins in transgenic *Arabidopsis* was performed in roots from 6-d-old seedlings grown in vertical position on Murashige and Skoog medium supplemented with 1% Suc and solidified with 0.8% (w/v) agar. Heat treatment was performed by transferring seedlings to 37°C for 2 h. Treatment with cycloheximide was performed by incubating seedlings in liquid Murashige and Skoog medium supplemented with 200 µg/mL of cycloheximide for 2 h at 37°C. Transient expression of fusion proteins in leaves of 3-week-old plants of *N. benthamiana* was assayed 3 d after agroinfiltration as described above. Microscopy images were collected using a TCS SP2 confocal laser spectral microscope (Leica Microsystems). The excitation lines for imaging GFP and RFP fusions were 488 and 561 nm, respectively.

Cordycepin Treatments, mRNA Half-Life Estimations, and Capped mRNA Analysis

Six-day-old seedlings and 2-week-old plants were used for cordycepin treatment, essentially as described (Gutiérrez et al., 2002). Samples were collected at the indicated time points, and total RNA was extracted using the Purezol reagent (Bio-Rad). Gene expression was analyzed by RNA blot hybridizations or real-time RT-PCR as described above. To examine U6 snRNA, U3 snoRNA, and U4 snRNA decay, additional cordycepin was added to the samples at 9 and 24 h to ensure transcriptional repression. For graphical representation of mRNA stability and mRNA half-life estimation, the hybridization bands were quantified with the ImageJ software (NIH), and values were normalized to wild-type time 0.

To determine if accumulating mRNAs were capped, RNA ligase–mediated RACE was performed using the First Choice RLM-RACE kit (Ambion) following the manufacturer's specifications. RNAs were extracted from 6-d-old seedlings or 2-week-old plants with the RNeasy kit (Qiagen), and PCRs were performed using a low (20 to 25) or high (30 to 32) number of cycles. Specific primers for the 5′ RACE adapter and for the genes tested are described in Supplemental Data Set 2D online.

Microarray Analysis

Total RNA from 2-week-old Col-0, *lsm1a lsm1b*, and *lsm8-1* plants was extracted using the RNeasy kit (Qiagen), and three biological replicates were independently hybridized per transcriptomic comparison. For microarray analysis of the *lsm1a lsm1b* mutant, RNA amplification and labeling were performed basically as described (Goda et al., 2008). Hybridization was performed on Agilent *Arabidopsis* Oligo Microarrays v4 (catalog number G2519F-V4021169) in accordance with the manufacturer's specifications. The statistical significance of the results was evaluated with FIESTA software (http://bioinfogp.cnb.csic.es). Genes with an false discovery rate–corrected P value lower than 0.05 and a fold change of more or less than 2 were selected for consideration. Data from these microarray experiments have been deposited in the Gene Expression Omnibus database under accession number GSE39630.

For microarray analysis of the *lsm8-1* mutant, double-stranded cDNAs were synthesized, processed, and labeled with the GeneChip whole-transcript double-stranded target assay kit (Affymetrix) following the manufacturer's instructions. Labeled cDNAs were used to hybridize Affymetrix *Arabidopsis* Tiling 1.0R arrays (catalog number 900594). Data were analyzed with Tiling Analysis Software from Affymetrix using TAIR7 as reference annotation (BPMAP file). To detect altered gene expression, genes with at least one exon identified as significantly over- or under-expressed (P value lower than 0.05 and a fold change of more or less than 2) were considered. A gene was accepted as differentially expressed when the 10% trimmed mean of the signals of all probes in its exons and UTRs was at least twofold higher or lower in the mutant than in the wild type. For those genes with splicing variants, only the constitutive exons were considered. Similarly, introns with significantly higher signals in the mutant than in the wild type were initially considered to be intron retention events. For high confidence, only the introns covered with a minimum of three probes and average signals over twofold were selected (see Supplemental Data Set 2A online). Data from these microarray experiments have been deposited in the Gene Expression Omnibus database under accession number GSE39617.

Intron Retention Analysis

Total RNA from 2-week-old plants was extracted with Purezol (Bio-Rad) and used for cDNAs generation with the iScript cDNA synthesis kit (Bio-Rad). Intron retention was revealed by RT-PCR using a pair of specific primers for each gene tested (see Supplemental Data Set 2D online). One primer was situated inside the retained intron and the second one in an adjacent exon. All PCR reactions were performed using RNA with (+RT) or without (−RT) reverse transcriptase to detect genomic DNA contaminations. Genomic DNA was included in all reactions as a positive control, and *TUBULIN* expression level was used as a loading control.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL data libraries under the accession numbers listed in Supplemental Data Set 2E online. The microarray data were submitted to the Gene Expression Omnibus site (www.ncbi.nlm.nih.gov/geo) under accession numbers GSE39630 and GSE39617.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Sequence Alignment of *Arabidopsis* LSM Proteins.

Supplemental Figure 2. Phylogenetic Analysis of Plant LSM Proteins.

Supplemental Figure 3. Expression Patterns of *LSM1A* and *LSM1B* Genes.

Supplemental Figure 4. Visualization of in Vivo Interactions between *Arabidopsis* LSM Proteins by BiFC Assays.

Supplemental Figure 5. Phenotypic Analysis of *lsm1a* and *lsm1b* Single Mutants.

Supplemental Figure 6. Quantification of Developmental Phenotypes Shown by *lsm* Mutants.

Supplemental Figure 7. Complementation of the *lsm1a lsm1b* Double Mutant by LSM1B.

Supplemental Figure 8. mRNA Stability and Accumulation of Capped Transcripts in c-*lsm1b*, *lsm1a*, *lsm1b*, and *lsm8-1* Plants.

Supplemental Figure 9. Tiling Array Hybridization Signals in Representative Genes Showing Intron Retention Events in the *lsm8-1* Mutant.

Supplemental Figure 10. Stability of U6 snRNA in the *lsm1a lsm1b* Double Mutant.

Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis Shown in Supplemental Figure 2.

Supplemental Data Set 2A. Intron Retention Events in the *lsm8-1* Mutant.

Supplemental Data Set 2B. Genes Whose Expression Is Up- or Downregulated in the *lsm1a lsm1b* Double Mutant.

Supplemental Data Set 2C. Genes Whose Expression Is Up- or Downregulated in the *lsm8-1* Mutant.

Supplemental Data Set 2D. Oligonucleotide Sequences of Primers Used in This Study.

Supplemental Data Set 2E. Accession Numbers of the Sequence Data Presented in This Study.

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AUTHOR CONTRIBUTIONS

C.P.-R. and T.H.-V. designed the research, performed research, and analyzed data. R.L.-C. and M.M.C. performed research. J.S. designed the research, analyzed data, and wrote the article.

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The LSM1-7 Complex Controls Plant Adaptation to Adverse Environmental Conditions by Promoting Selective mRNA Decapping

Carlos Perea-Resa, Cristian Carrasco-López, Rafael Catalá, Veronika Turečková, Ondrej Novak, Weiping Zhang, Leslie E. Sieburth, José M. Jiménez-Gómez and Julio Salinas

Departamento de Biología Medioambiental, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain

Posttranscriptional regulation of gene expression is crucial for plant adaptation to abiotic stresses. Yet, the operating mechanisms, their components and their modes of action are poorly understood. Here, we report that the Arabidopsis LSM1-7 decapping activator complex plays a critical role in plant tolerance to adverse environmental conditions. Our results show that, depending on the condition, the complex interacts with different stress-inducible transcripts targeting them for decapping and subsequent degradation. This interaction ensures the correct turnover of the target transcripts and, consequently, the appropriate patterns of downstream stress-responsive gene expression that are required for plant adaptation. Remarkably, among the target transcripts we found are those encoding NCED3 and NCED5, two key enzymes in ABA biosynthesis. We demonstrate that the LSM1-7 complex modulates the levels of ABA in plants exposed to abiotic stresses by differentially controlling *NCED3* and *NCED5* mRNAs turnover, which represents a new layer of regulation in ABA biosynthesis.

INTRODUCTION

Given their sessile nature, plants are continuously challenged by numerous abiotic stresses such as low temperature, drought, or salinity. These environmental factors negatively affect plant growth and development, and result in reduced productivity and significant crop losses. Plants have evolved sophisticated mechanisms to perceive and rapidly respond to abiotic stresses. A decisive component of all stress responses is the ability to reprogram transcriptomes. Phytohormones, specially the abscisic acid (ABA), play a central role in regulating abiotic stress-responsive gene expression. Cold, water and salt stresses promote an increase in ABA levels, mainly as a result of *de novo* biosynthesis, that is subsequently transduced to induce a plethora of genes required for correct plant adaptation (Finkelstein, 2013). It is generally assumed that the reaction catalyzed by 9-cis-epoxycarotenoid dioxigenase (NCED) is the rate-limiting step in ABA biosynthesis (Nambara and Marion-Poll, 2005). Five NCED encoding genes have been characterized in Arabidopsis, *NCED3* and, to a lesser extent, *NCED5* contributing to ABA biosynthesis in response to abiotic stresses (Tan et al., 2003; Frey et al., 2012). The regulation of *NCED3* and *NCED5* expression by these adverse conditions, however, is poorly characterized. It has been described that *NCED3* transcripts accumulate by different stress treatments and by exogenous ABA, indicating a positive feedback regulation (Cuevas et al., 2008; Frey et al., 2012; Yang and Tan, 2014). This accumulation would be regulated at the transcriptional level (Jiang et al., 2012; Yang and Tan, 2014).

Recent data indicate that posttranscriptional mechanisms are also influential to regulate messenger availability under stress situations and, therefore, to finely and timely adjust transcriptomes to unfavorable environments (Guerra et al., 2015). The control of mRNA stability, in particular, is critical for the regulation of gene expression in response to abiotic stress. Stability determinants for intrinsically unstable eukaryotic mRNAs include the 3' poly (A) tail and the 5' cap. Transcript abundance is then fine-tuned by a major pathway of cytoplasmic mRNA degradation involving deadenylation, decapping and subsequent exoribonuclease 5'-3' activity (Parker, 2012). Unfortunately, the role of this mRNA degradation pathway in plant response to abiotic stress is barely documented. AtCAF1a, a component of the carbon catabolite repressor 4-CCR4 associated factor 1 (CCR4-CAF1) complex that serves as the major deadenylase complex in Arabidopsis, was described to negatively regulate germination under high salt conditions, likely by targeting unique messengers for deadenylation (Walley et al., 2010). Arabidopsis decapping activators DCP1 and DCP5 associate to promote drought tolerance through the decapping of a subset of dehydration-responsive genes (Xu and Chua, 2012). Finally, XRN4, the only cytoplasmic exoribonuclease in Arabidopsis, and its cofactor LARP1 were shown to be necessary for plant tolerance to moderate high temperature by inducing a heat-triggered global mRNA decay (Merret et al., 2013). These and additional results from yeast and animal studies (Castells-Roca et al., 2011; Nousch et al., 2013; Watanabe et al., 2013) indicate that different components of the 5'-3' mRNA degradation pathway can regulate responses to different abiotic stresses. Whether a given component may be implicated in controlling different responses remains unknown.

The SM-like proteins (LSMs) are implicated in numerous aspects of RNA metabolism in eukaryotes. We previously reported that the Arabidopsis genome contains eleven *LSM* genes encoding eight central, highly evolutionarily conserved LSM proteins (LSM1-LSM8) (Perea-Resa et al., 2012). Genes *LSM1*, *LSM3* and *LSM6* are duplicated and code for pairs of functionally redundant proteins (LSM1A, B; LSM3A, B; LSM6A, B). In Arabidopsis, as in yeast and animals, the eight conserved LSM proteins are organized in two heteroheptameric ring complexes, LSM1-7 and LSM2-8, specifically localized in the cytoplasm and nucleus, and defined by the subunits LSM1 and LSM8, respectively. The LSM2-8 complex functions in pre-mRNA splicing through U6 snRNA stabilization, and ensures normal Arabidopsis development (Perea-Resa et al., 2012). Moreover, alternative splicing analysis in *lsm4* and *sad1*/*lsm5* mutants uncovered that this complex acts as a positive regulator of salt tolerance in Arabidopsis (Zhang et al., 2011; Cui et al., 2014). The LSM1-7 complex is involved in accurate mRNA turnover by

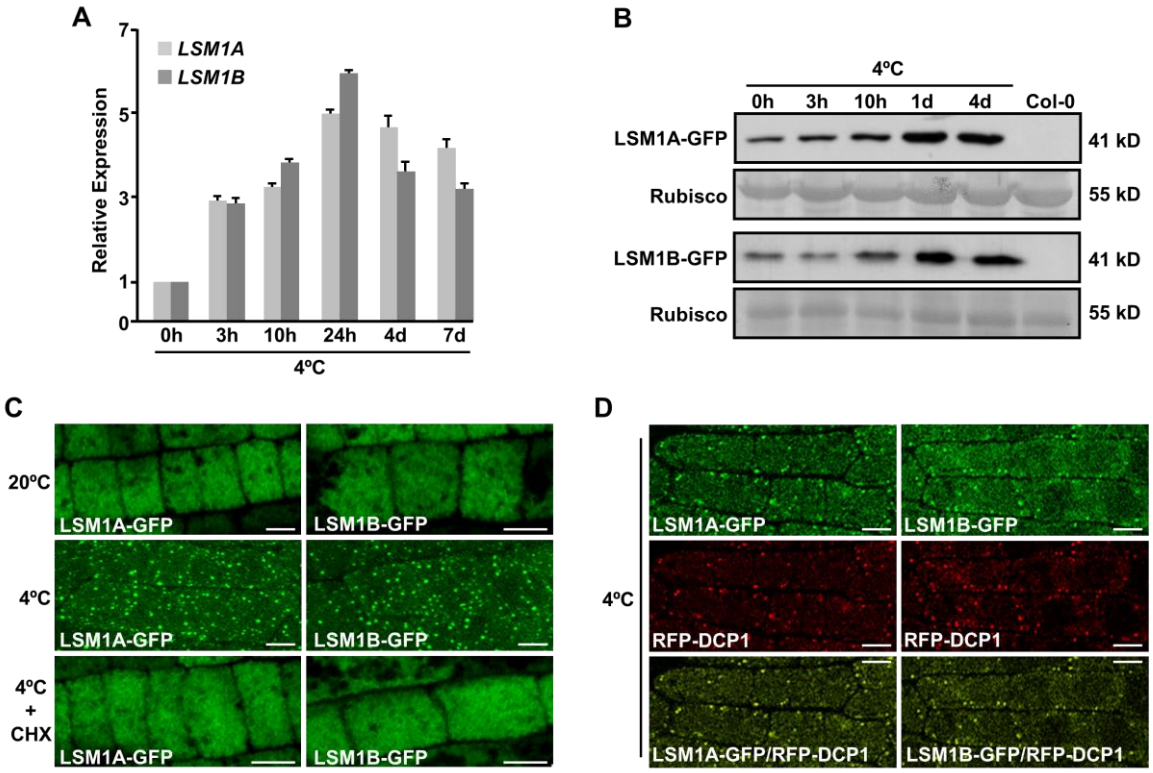

Figure 1. Arabidopsis LSM1 proteins accumulate in response to low temperature and localize to P-bodies.
(A) Expression of *LSM1A* and *LSM1B* in 2-week-old Col-0 plants exposed for the indicated hours (h) or days (d) to 4ºC. Levels, determined by qPCR, are represented as relative to their corresponding values at 0h. Error bars indicate SD.
(B) Levels of LSM1A-GFP and LSM1B-GFP in 2-week-old transgenic Arabidopsis plants exposed for the indicated times to 4ºC. A lane with Col-0 plants was added in the western blot as a negative control. The large subunit of Rubisco was used as a loading control.
(C) Subcellular localization of LSM1A-GFP and LSM1B-GFP in root tip cells from 6-day-old transgenic Arabidopsis seedlings grown under control conditions (20ºC), exposed 24h to 4ºC, or exposed 24h to 4ºC with cycloheximide (CHX). Bars = 20 µm.
(D) Colocalization of LSM1A-GFP and LSM1B-GFP with RFP-DCP1 in root tip cells from 6-day-old transgenic Arabidopsis seedlings exposed 24h to 4ºC. Bars = 20 µm.

promoting decapping and subsequent 5'-3' degradation, and is required for the formation of processing bodies (P-bodies), the cytoplasmic foci where mRNA decay occurs. It is, therefore, part of the Arabidopsis decapping apparatus along with the catalytic component DCP2 and its other activators (i.e., DCP1, DCP5, VARICOSE and PAT1) (Perea-Resa et al., 2012; Roux et al., 2015). In yeast, the LSM1-7 complex has been described to operate through the interaction of LSM1 with oligoadenylated mRNAs that are then targeted for degradation (Chowdhury et al. 2007). The analysis of an Arabidopsis *lsm1a lsm1b* double mutant defective in *LSM1A* and *LSM1B* expression unveiled that LSM1 proteins are essential for the assembly of the LSM cytoplasmic complex and that this complex is needed for correct Arabiodopsis development (Perea-Resa et al., 2012). The implication of the LSM1-7 decapping activator complex, however, in plant responses to abiotic stresses has not yet been established. Here, we demonstrate that this complex regulates Arabidopsis tolerance to freezing, drought and high salt by modulating the transcriptome reprogramming that take place in response to these adverse conditions. More important, RNA immunoprecipitation (RIP) assays revealed that, depending on the abiotic stress to which the plant is subjected, the LSM cytoplasmic complex targets selected stress-inducible transcripts for decapping and degradation, thus controlling their levels and, therefore, ensuring the adequate transcriptomic response. Interestingly, among the selected mRNAs that are differentially targeted for decapping in response to low temperature, water deficiency and salinity are *NCED3* and *NCED5*. We show that, as a consequence, the LSM1-7 complex determines the appropriate levels of ABA in Arabidopsis plants exposed to different abiotic stresses, which represents a new layer of regulation in the biosynthesis of this phytohormone under unfavorable environmental situations.

RESULTS

Arabidopsis LSM1 proteins localize to P-bodies in response to abiotic stress

The molecular characterization of Arabidopsis *LSM1* genes uncovered that they were responsive to low temperature. Quantitative RT-PCR (qPCR) assays showed that *LSM1A* and *LSM1B* transcripts accumulated in response to 4°C, reaching a peak of accumulation after 1 day of treatment (Figure 1A). This accumulation was detected in all organs of adult Arabidopsis (Figure S1A). Transcripts corresponding to LSM2-7 proteins exhibited similar cold-induced accumulation as *LSM1* mRNAs (Figure S1B). *LSM* transcripts did not accumulate, however, in plants exposed to other related abiotic stresses, such as drought (55% PEG) or high salt (150mM NaCl) (Figure S1C). We

concluded that the expression of genes encoding the Arabidopsis cytoplasmic LSM complex is positively regulated by low temperature.

Since *LSM1A* and *LSM1B* transcripts accumulated in response to low temperature, we assessed whether this accumulation was followed by an increase of the corresponding proteins. Western-blot experiments using Arabidopsis plants containing genomic *LSM1-GFP* fusions driven by the corresponding *LSM1* promoters ($LSM1_{PRO}$) (Perea-Resa et al., 2012) displayed that LSM1A-GFP and LSM1B-GFP proteins were notably more abundant after some days of cold exposure (Figure 1B). Consistent with the expression results, water and salt stresses did not alter the levels of LSM1 proteins (Figure S2A). Therefore, concomitantly with the accumulation of their transcripts, the levels of LSM1 proteins also increase in response to low temperature.

Our previous data revealed that heat treatment promotes the localization of LSM1A and LSM1B to P-bodies (Perea-Resa et al., 2012). Then, we decided to investigate if LSM1 proteins also localized to these foci under cold, drought or high salt conditions by examining the distribution of green fluorescence in root cells from the $LSM1A_{PRO}$-*LSM1A-GFP* and $LSM1B_{PRO}$-*LSM1B-GFP* plants. According to our earlier results (Perea-Resa et al., 2012), confocal microscopy analysis indicated that, at 20°C, GFP activity was in both cases evenly distributed in the cytoplasm (Figure 1C). When transgenic plants were exposed to 4°C, LSM1A-GFP and LSM1B-GFP fusion proteins were found, however, aggregated in discrete cytoplasmic spots that resembled P-bodies. After cycloheximide treatment, which causes loss of P-bodies, no cytoplasmic foci were observed, suggesting that the detected LSM1-GFP spots indeed corresponded to P-bodies (Figure 1C). The identity of these foci was confirmed by colocalization studies with DCP1, a protein that localizes to P-bodies (Motomura et al., 2015). The examination of $LSM1A_{PRO}$-*LSM1A-GFP* and $LSM1B_{PRO}$-*LSM1B-GFP* plants cotransformed with a *35S-RFP-DCP1* fusion (Perea-Resa et al., 2012) unveiled that, in fact, LSM1A-GFP and LSM1B-GFP colocalized with RFP-DCP1 in root cells exposed to 4°C (Figure 1D). Identical results were obtained when $LSM1A_{PRO}$-*LSM1A-GFP* and $LSM1B_{PRO}$-*LSM1B-GFP* plants were subjected to water and salt stresses (Figure S2B and S2C), evidencing that LSM1 proteins localize to P-bodies in response to low temperature, drought and high salt.

The LSM1-7 complex regulates Arabidopsis tolerance to abiotic stress

To further characterize the role of LSM1 proteins and, therefore, of the LSM cytoplasmic complex in plant adaptation to abiotic stress, we evaluated their possible implication in Arabidopsis tolerance to freezing, drought and salinity. Freezing tolerance was analyzed in non-acclimated and cold acclimated (7d, 4°C) *lsm1a lsm1b* mutants exposed 6h to different freezing temperatures. Non-acclimated mutants presented a similar capacity to tolerate freezing as the WT, the LT_{50} (temperature that causes 50% lethality) values being in both cases around -4.5°C (Figures S3A and S3B). In contrast, the freezing tolerance of cold acclimated *lsm1a lsm1b* mutants was significantly higher than that of WT plants. In this case, the determined LT_{50} values were -11°C and -9°C, respectively (Figure 2A).

Water stress tolerance was examined in *lsm1a lsm1b* seedlings transferred to plates containing 25% PEG. After one week, they exhibited a significant enhanced tolerance compared with WT seedlings as revealed by the quantification of their fresh weights and lateral roots (Figure 2B). Tolerance to high salt was assayed in *lsm1a lsm1b* seedlings growing one further week on plates containing 150mM NaCl. Mutants displayed lower fresh weights and shorter main roots than WT seedlings (Figure 2C), manifesting more sensitivity to salt stress. It is worth noting that *lsm1a lsm1b* plants grown on soil showed identical drought and salt tolerance phenotypes as those described for plate-growing seedlings (Figures S3B and S3C).

In all cases, *lsm1a lsm1b* mutants complemented with either $LSM1A_{PRO}$-*LSM1A-GFP* (*c-lsm1a*) or $LSM1B_{PRO}$-*LSM1B-GFP* (*c-lsm1b*) fusions had WT capacity to cold acclimate and to tolerate water deficiency and high salt (Figure 2; Figures S3A-C), confirming our earlier results on the functional redundancy of LSM1A and LSM1B (Perea-Resa et al., 2012), and establishing that the stress tolerance phenotypes of *lsm1a lsm1b* were a direct consequence of the absence of *LSM1A* and *LSM1B* expression. All these data provided genetic evidence that the LSM1-7 complex is differentially involved in plant tolerance to abiotic stresses. It negatively regulates the ability of Arabidopsis to cold acclimate and tolerate drought, but functions as a positive regulator of salt tolerance.

The Arabidopsis LSM cytoplasmic complex differentially regulates gene expression in response to abiotic stress

Since the Arabidopsis LSM1-7 complex controls gene expression by promoting RNA decapping and decay (Perea-Resa et al., 2012), we examined whether it might control gene expression in response to abiotic stresses as a first step to understand how it differentially regulates plant tolerance to environmental challenges. High-throughput RNA sequencing (RNAseq) was used to estimate the impact of *lsm1a* and *lsm1b* mutations on the transcriptomes of Arabidopsis plants subjected to cold, drought or high salt conditions. For this, we sequenced cDNA libraries prepared from stress-treated *lsm1a lsm1b* and WT plants. The resulting reads (±12 Mbp/sample) were mapped to the Arabidopsis genome (TAIR10 version) and gene expression changes in the double mutant evaluated. In each treatment, the top 1000 upregulated and top 1000 downregulated genes in *lsm1a lsm1b*, based on fold change ratios with respect to their corresponding controls, were considered for analysis. The expression levels of the top 1000 upregulated genes in mutant plants exposed 24h to 4°C were increased at least 3.4-fold compared with the WT (Table S1). Remarkably, 53.3% of these genes (i.e., 533) had been reported to be induced (≥2 fold) in response to cold (Kilian et al., 2007) (Table S2). Many of them, moreover, had been associated with the development of cold acclimation (Miura and Furumoto, 2013; Shi et al., 2015) and, therefore, should account for the tolerant phenotype of the double mutant. The upregulation of some of these genes in *lsm1a lsm1b* was verified by qPCR, validating the RNA-seq results (Figure 3A). Compared with the WT, the top 1000 downregulated genes in cold-treated mutants displayed a decreased expression of at least 2.1-fold (Table S3). Of them, 311 (i.e., 31.1%) had been described to be cold-induced (Kilian et al., 2007) (Table S4), but none functioning as negative regulator of cold acclimation.

Under conditions of water deficiency (10h, 55% PEG), the transcript levels of the top 1000 upregulated genes in *lsm1a lsm1b* plants were found to be higher, by at least 2-fold, than in the WT (Table S5). Interestingly, 372 of these genes (i.e., 37.2%) had been shown to be induced (≥2 fold) in response to drought (Kilian et al., 2007) (Table S6) and some of them, such as *ERF53*, *ABR1* or *ANAC019* to have a positive role in Arabidopsis tolerance to water stress (Tran et al., 2004; Pandey et

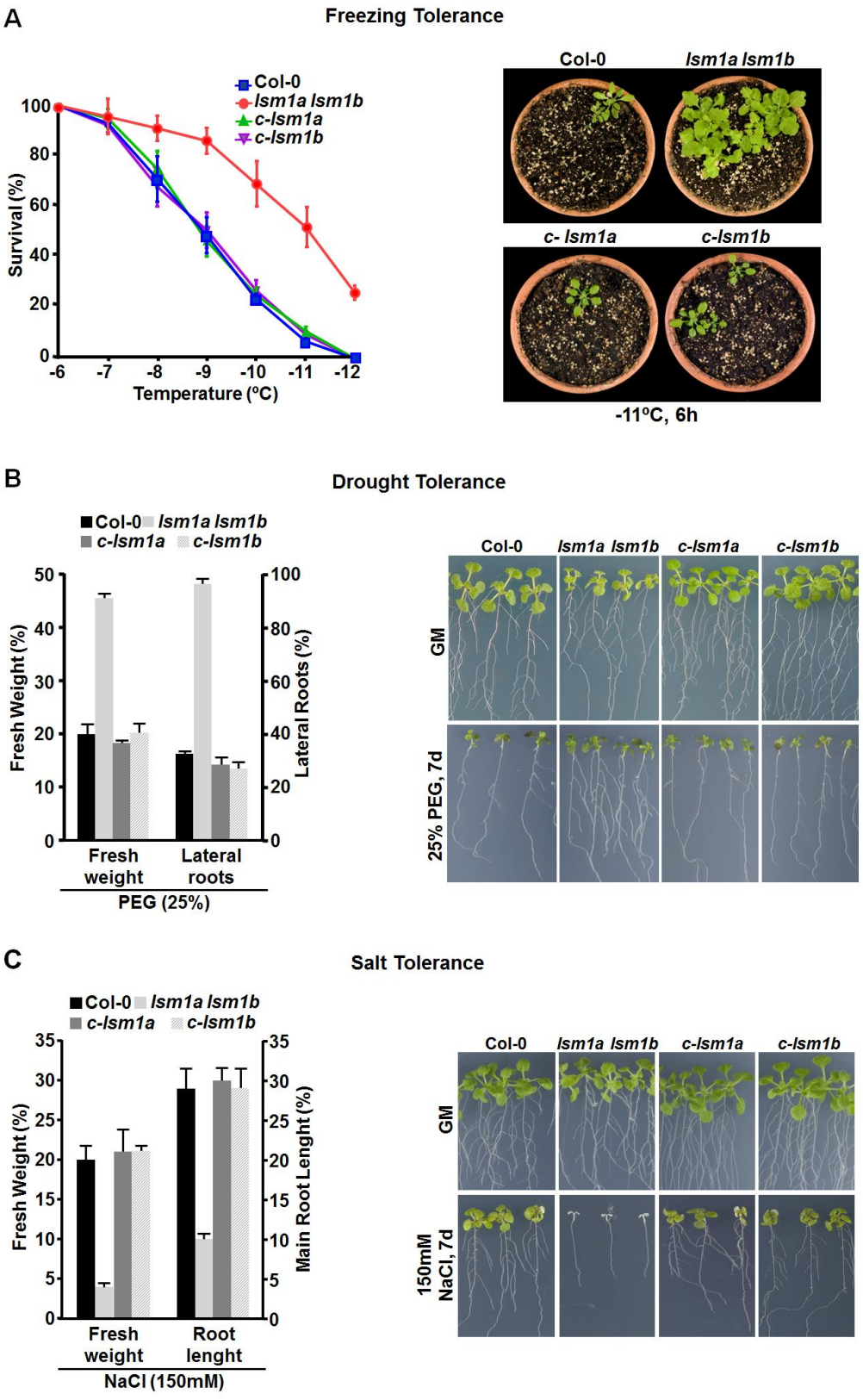

Figure 2. The LSM1-7 complex differentially regulates abiotic stress tolerance in Arabidopsis.
(A) Freezing tolerance of cold acclimated 2-week-old Col-0, *lsm1a lsm1b*, *c-lsm1a* and *c-lsm1b* plants. Error bars indicate SD (left). Representative cold acclimated plants 7d after being exposed to -11ºC for 6h (right).
(B) Drought tolerance of 7-day-old Col-0, *lsm1a lsm1b*, *c-lsm1a* and *c-lsm1b* seedlings. Error bars indicate SD (left). Representative seedlings grown on GM or exposed 7d to 25% PEG (right).
(C) Salt tolerance of 7-day-old Col-0, *lsm1a lsm1b*, *c-lsm1a* and *c-lsm1b* seedlings. Errors bars indicate SD (left). Representative seedlings grown on GM or exposed 7d to 150mM NaCl (right).

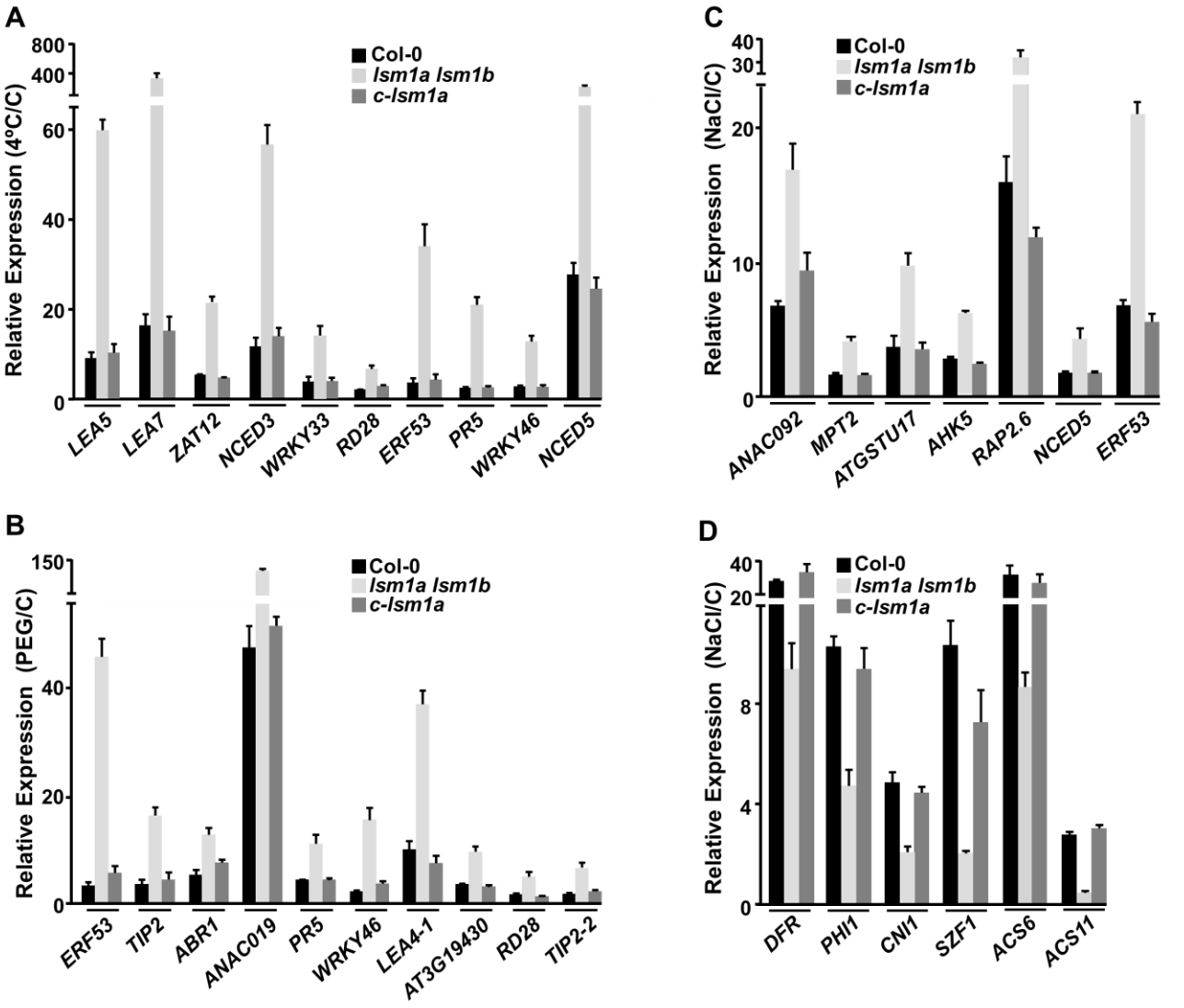

Figure 3. Arabidopsis LSM1-7 complex differentially regulates gene expression in response to abiotic stresses.

(A) Expression of different cold-inducible genes up-regulated in *lsm1a lsm1b*. Levels, determined by qPCR, in 2-week-old Col-0, *lsm1a lsm1b* and *c-lsm1a* plants exposed 24h to 4ºC are represented as relative to their corresponding values in control plants (C). Error bars indicate SD.

(B) Expression of different drought-inducible genes up-regulated in *lsm1a lsm1b*. Levels, determined by qPCR, in 2-week-old Col-0, *lsm1a lsm1b* and *c-lsm1a* plants exposed 10h to 55% PEG are represented as relative to their corresponding values in control plants (C). Error bars indicate SD.

(C-D) Expression of different salt-inducible genes up- **(C)** or down-regulated **(D)** in *lsm1a lsm1b*. Levels, determined by qPCR, in 2-week-old Col-0, *lsm1a lsm1b* and *c-lsm1a* plants exposed 10h to 150mM NaCl are represented as relative to their corresponding values in control plants (C). Error bars indicate SD.

al., 2005; Cheng et al., 2012). qPCR experiments confirmed the upregulation of these and other drought-inducible genes in *lsm1a lsm1b* plants (Figure 3B), which would account for the drought tolerant phenotype of the mutants and would validate the RNA-seq data. Otherwise, the top 1000 downregulated genes in mutant plants subjected to water stress exhibited at least 1.9-fold lower expression levels than in WT plants (Table S7). The 32.6% of them (i.e., 326) had been disclosed to be induced by water deficiency (Kilian et al., 2007) (Table S8), although none acting as negative regulator of drought tolerance.

When comparing the transcriptome profiles from mutant and WT plants exposed to salt stress (10h, 150mM NaCl), the expression levels of the top 1000 upregulated genes in *lsm1a lsm1b* were at least 2.4-fold higher than in the WT (Table S9). In this case, 47% of the genes (i.e., 470) had been reported to be salt-induced (≥2-fold) (Kilian et al., 2007) (Table S10), and several of them, such as *ANAC092*, *ATGSTU17* or *AHK5,* to act as negative regulators of salt tolerance in Arabidopsis (Balazadeh et al., 2010; Pham et al., 2012; Chen et al., 2012). The upregulation of these and other salt-inducible genes was verified by qPCR, validating the RNAseq data (Figure 3C). The top 1000 downregulated genes in mutant plants showed at least 2.1-fold lower expression levels than in the WT (Table S11). Strikingly, 457 (i.e., 45.7%) of these genes were salt-induced (Kilian et al., 2007) (Table S12) and some of them, including *DFR*, *PHI1* or *CNI1* had been described to be positive regulators of Arabidopsis tolerance to high salt (Cui et al., 2014; Peng et al., 2014). qPCR experiments confirmed the downregulation of these and other salt-inducible genes in salt-treated *lsm1a*

lsm1b plants, validating once again the RNA-seq data (Figure 3D). The altered expression of the negative and positive regulators of salt tolerance mentioned above was fully consistent with the salt sensitive phenotype exhibited by the double mutant.

It should be noted that, according to the close relationship existing between plant responses to low temperature, water deficiency and high salt (Kilian et al., 2007), several genes were regulated by the LSM1-7 complex in response to more than one stress condition. Nonetheless, most LSM1-7 regulated genes resulted to be stress specific (Figure S4, Tables S2, S6 and S10). In any case, *c-lsm1a* plants presented WT expression patterns for all validated genes (Figures 3A-D). On the whole, these results indicated that the Arabidopsis LSM cytoplasmic complex regulates Arabidopsis tolerance to abiotic stresses by differentially controlling stress-responsive gene expression.

The Arabidopsis LSM1-7 complex regulates the turnover of stress-dependent selected transcripts in response to abiotic stresses

Considering the capacity of the LSM cytoplasmic complex to control transcript turnover through the interaction of LSM1 with target mRNAs, promoting their decapping and subsequent degradation (Chowdhury et al., 2007), we reasoned that it could differentially regulate gene expression in response to abiotic stresses by promoting the decay of selected transcripts in a stress-dependent manner. To investigate this idea, we first identified direct targets of the complex under low temperature, drought and high salt conditions by means of RIP assays. These assays were performed with *c-lsm1a* plants grown under control conditions or exposed to 4°C, 55% PEG or 150mM NaCl. Transcripts interacting with LSM1A-GFP were co-immunoprecipitated (co-IP) with anti-GFP antibody and then identified by qPCR. Arabidopsis transgenic plants expressing a GFP-tagged GRP7 protein (Streitner et al., 2012) were also used in RIP experiments to control the specificity of the detected interactions. Given the function of the LSM1-7 complex, it was expected that some of the mRNAs up-regulated in the *lsm1a lsm1b* mutants exposed to abiotic stresses were direct targets in response to such conditions. Targets, therefore, were searched among the cold-, drought- and salt-inducible transcripts whose levels we had confirmed were up-regulated in *lsm1a lsm1b* mutants in response to low temperature, water stress and high salt, respectively (Figures 3A-3C). As anticipated, several cold-induced transcripts, including *LEA7*, *ZAT12*, *NCED3*, *WRKY33*, *RD28*, *ERF53*, *WRKY46*, and *NCED5* were significantly enriched in the co-IP RNA samples from cold-treated *c-lsm1a* plants but not in the RNA samples from *GRP7-GFP* plants (Figure 4A), evidencing that they were direct targets of the Arabidopsis LSM cytoplasmic complex in response to low temperature. Some mRNAs, i.e. *ZAT12*, *NCED3*, *RD28*, *ERF53*, and *NCED5*, were already significantly detected in the precipitated RNA samples recovered from unstressed *c-lsm1a* plants (Figure 4A), manifesting that, although with a lower affinity than that at 4°C, they were also targets of the complex under control conditions.

Compared to those obtained from *GRP7-GFP* plants, Co-IP RNA samples obtained from *c-lsm1a* plants exposed to water deficiency revealed a specific and significant enrichment in *ERF53, ABR1, ANAC019*, *WRKY46* and *RD28* transcripts (Figure 4B). Consistent with the results obtained when looking for targets of the complex under cold conditions (Figure 4A), *ERF53* and *RD28* mRNAs were also found to be significantly detected in the precipitated RNA samples from control *c-lsm1a* plants (Figure 4B). In the case of RNA samples collected from *c-lsm1a* and *GRP7-GFP* plants subjected to high salt, RIP assays uncovered a specific and significant enrichment of *ANAC092*, *AHK5*, *NCED5* and *ERF53* mRNAs in *c-lsm1a* samples (Figure 4C). *NCED5* and *ERF53* transcripts were also significantly detected in the co-IP RNA samples from non-stressed *c-lsm1a* plants (Figure 4C).

As anticipated from the function of the LSM cytoplasmic complex in mRNA degradation by promoting the decapping of its targets (Perea-Resa et al., 2012), rapid amplification of cDNA ends (RACE)-PCR experiments revealed that *LEA7*, *ZAT12*, *NCED3*, *WRKY33*, *RD28*, *ERF53*, *WRKY46*, and *NCED5* messengers accumulated in their capped forms in *lsm1a lsm1b* mutants compared with WT plants exposed to low temperature (Figure 5A). Additionally, the levels of capped *ZAT12*, *NCED3*, *RD28*, *ERF53*, and *NCED5* transcripts were higher in mutant than in WT plants grown under control conditions (Figure 5A). RACE-PCR experiments with RNAs from water-stressed *lsm1a lsm1b* and WT plants disclosed that capped *ERF53, ABR1, ANAC019*, *WRKY46* and *RD28* messengers were significantly increased in the mutants. Moreover, as expected, *ERF53* and *RD28* transcripts were also augmented in their capped forms in unstressed mutants (Figure 5B). Otherwise, the levels of capped *ANAC092*, *AHK5*, *NCED5* and *ERF53* mRNAs were clearly higher in *lsm1a lsm1b* than in WT plants exposed to NaCl (Figure 5C). Furthermore, capped *NCED5* and *ERF53* transcripts also accumulated in mutants grown under standard conditions (Figure 5C). The capped forms of all mRNAs analyzed were always found not to be altered in *c-lsm1a* plants (Figures 5A-C).

All these results strongly suggested that the LSM1-7 complex would control the turnover of different selected stress-responsive target transcripts, including both specific and non-specific ones, in response to different abiotic stresses. The existence of non-specific target mRNAs, such as *RD28*, *ERF53*, *WRKY46* and *NCED5*, has been already evidenced (Figures 4 and 5). The existence of specific targets was established by assessing the affinity of the complex for *ZAT12*, *LEA7*, *NCED3*, *WRKY33*, *ABR1*, *ANAC019*, *ANAC092* and *AHK5* transcripts under different unfavorable conditions. Thus, RIP experiments showed that *LEA7*, *ZAT12*, *NCED3* and *WRKY33*, direct targets of the LSM cytoplasmic complex in response to low temperature (Figures 4A and 5A), were not enriched in co-IP RNA samples from *c-lsm1a* plants exposed to water deficiency and high salt (Figure S5A). In turn, *ABR1* and *ANAC019*, direct targets of the complex in response to drought (Figures 4B and 5B), were not recovered with LSM1-GFP in response to cold and salt stresses (Figure S5B). Otherwise, *ANAC092* and *AHK5*, direct targets of the complex in response to high salt (Figures 4C and 5C), were found not to be enriched in precipitated RNA samples from *c-lsm1a* plants subjected to 4°C and water stress (Figure S5C). In addition, as expected from the RIP assays, the levels of capped *LEA7*, *ZAT12*, *NCED3* and *WRKY33* messengers did not increase in *lsm1a lsm1b* mutants under drought and high salt conditions (Figure S6A), and *ABR1* and *ANAC019* mRNAs did not accumulate in their capped forms in mutant plants in response to low temperature and salt stress (Figure S6B). Moreover, capped *ANAC092* and *AHK5* transcripts did not augment in *lsm1a lsm1b* in response to cold and water deficiency (Figure S6C). In all cases, *c-lsm1a* plants presented equivalent levels of capped mRNAs as WT plants (Figure S6).

Together, our data indicated that the Arabidopsis LSM1-7 complex differentially regulates gene expression in response to

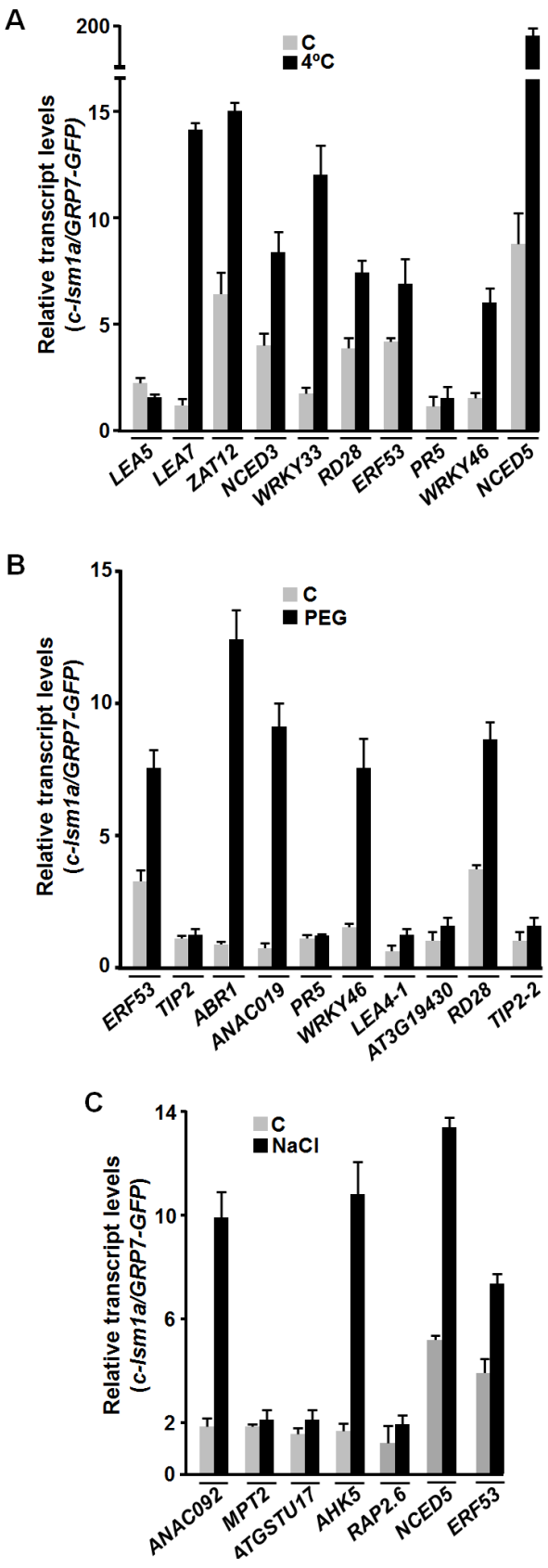

Figure 4. Arabidopsis LSM1-7 complex interacts with selected target transcripts in response to abiotic stresses.

(A-C) RIP assays on 2-week-old *c-lsm1a* plants grown under control conditions (C), exposed 24h to 4ºC **(A)**, 10h to 55% PEG **(B)**, or 10h to 150mM NaCl **(C)**, using an anti-GFP antibody. RIP assays on Arabidopsis containing a $GRP7_{PRO}$-*GRP7-GFP* fusion grown under control and stressed conditions were also carried out as interaction specificity controls. Coimmunoprecipitated RNA samples corresponding to different cold- **(A)**, drought- **(B)** and salt-inducible **(C)** genes were quantified by qPCR. Transcript levels in *c-lsm1a* plants were corrected with respect to their corresponding input values and represented relative to the levels obtained from RIP control assays. Error bars indicate SD.

abiotic stresses, and consequently plant tolerance to these challenging situations, by controlling in each case the decay of selected, both specific and non-specific, stress-responsive transcripts. The fact that some of these mRNAs are, furthermore, direct targets of the complex at 20°C, suggests that it should also regulate stress-responsive gene expression under control conditions.

The Arabidopsis LSM cytoplasmic complex regulates ABA biosynthesis in response to abiotic stress

Interestingly, among the mRNAs whose decay is differentially regulated by the Arabidopsis LSM1-7 complex in response to abiotic stresses are those encoding NCED3 and NCED5. In fact, while both *NCED3* and *NCED5* transcripts are direct targets of the complex under low temperature conditions (Figures 4A and 5A), their levels are not specifically affected in water-stressed *lsm1a lsm1b* plants (Table S5) and, as anticipated, none of them are targets of the complex in response to drought (Figure S7). Under salt stress, however, the *NCED5* transcript, but not the *NCED3* one, is upregulated in salt-treated mutants (Figure 3C and Table S9) and is a direct target of the LSM cytoplasmic complex (Figures 4C, 5C and S7). These data strongly suggested that the LSM1-7 complex should differentially regulate ABA biosynthesis in Arabidopsis in response to abiotic stresses. To test this prediction, we measured the ABA content of WT and *lsm1a lsm1b* mutant plants grown under control conditions or exposed to low temperature (4°C), water deficiency (55% PEG), or high salt (150mM NaCl). When exposed to low temperature, ABA levels increased in both WT and *lsm1a lsm1b* mutants but the increase was significantly higher in mutants than in WT plants (Figure 6A). Unstressed *lsm1a lsm1b* mutants also showed higher levels of ABA than WT plants (Figure 6A). These levels, however, were much lower than those detected after cold treatment, paralleling the lower binding affinity of the LSM cytoplasmic complex for *NCED3* and *NCED5* mRNAs at 20°C compared with 4°C (Figures 4A and 5A). *c-lsm1a* plants always exhibited similar ABA content as WT plants (Figure 6A), evidencing that the Arabidopsis LSM1-7 complex attenuates ABA biosynthesis in response to low temperature and, to a lesser extent, under standard conditions by promoting the decay of *NCED3* and *NCED5* messengers.

ABA levels also increased very prominently in Arabidopsis WT plants exposed to water stress or high salt (Figure 6B). In the case of *lsm1a lsm1b* mutants, water deficiency caused an accumulation of ABA similar to that of WT plants. In contrast, salt stress provoked an accumulation of ABA significantly higher than in WT plants. *c-lsm1a* plants exhibited similar ABA content as the WT in response to both water and salt stresses (Figure 6B). Therefore, as predicted from the expression and RIP analyses, the LSM cytoplasmic complex also controls the biosynthesis of ABA in Arabidopsis plants exposed to salt stress but it is not involved in ABA biosynthesis in response to water stress. Collectively, these findings demonstrated that the Arabidopsis LSM1-7 complex guarantees the adequate levels of

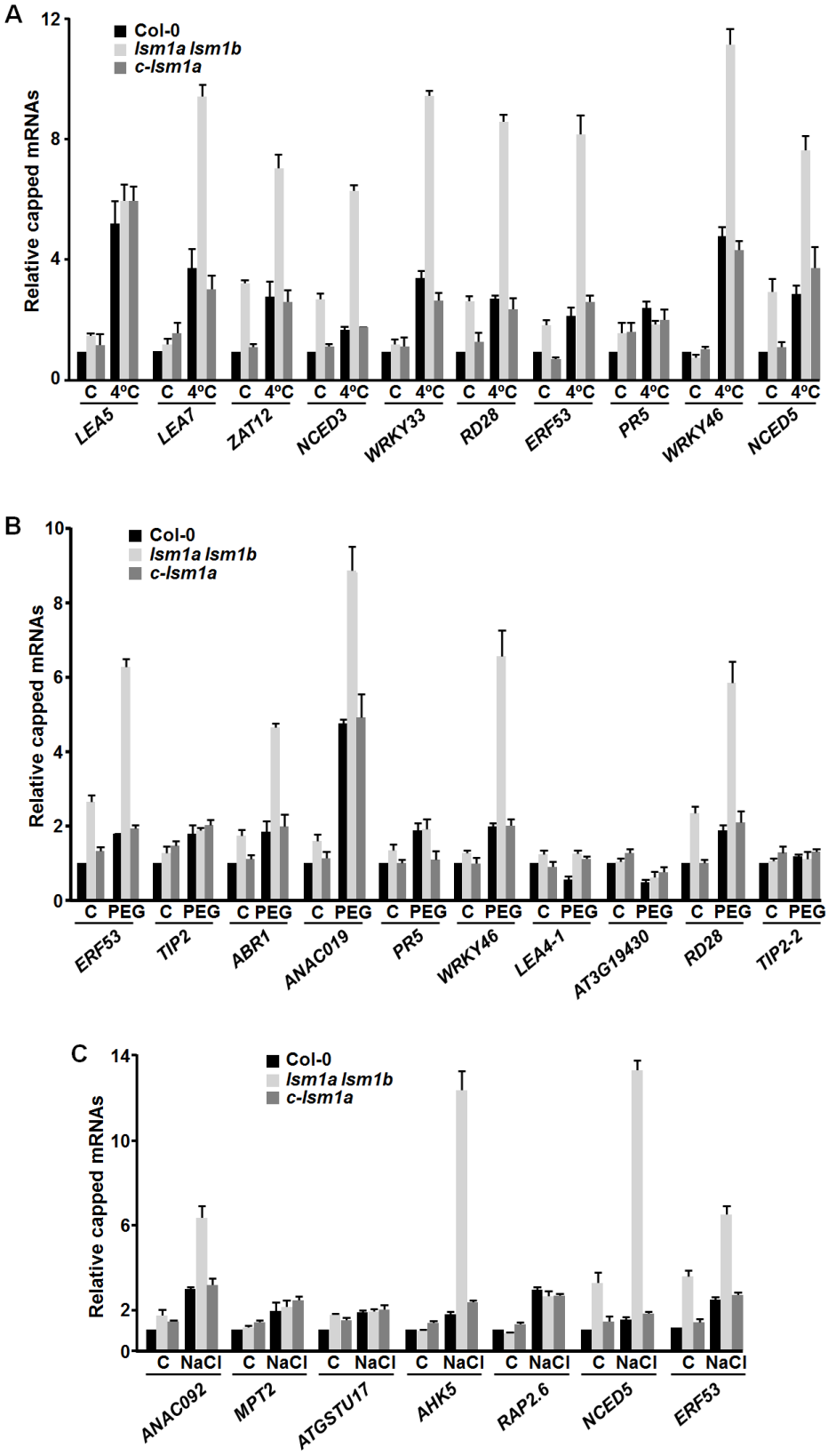

Figure 5. Arabidopsis LSM1-7 complex promotes decapping of selected target transcripts in response to abiotic stresses.

(A-C) Capped transcripts in 2-week-old Col-0, *lsm1a lsm1b* and *c-lsm1a* plants grown under control conditions (C), exposed 24h to 4ºC **(A)**, 10h to 55% PEG **(B)**, or 10h to 150mM NaCl **(C)**. The levels of capped transcripts corresponding to different cold- **(A)**, drought- **(B)** and salt-inducible **(C)** genes were corrected with respect to the levels of their corresponding total transcripts and represented relative to control Col-0 plants. Error bars indicate SD.

ABA in Arabidopsis plants exposed to different abiotic stresses by differentially controlling the decay of *NCED3* and *NCED5* transcripts.

DISCUSSION

The control of mRNA stability is a key step in the regulation of gene expression. However, the impact of mRNA turnover on plant transcriptome reprogramming in response to abiotic stress, and its importance to stress tolerance are largely unknown. Here, we show that the Arabidopsis LSM1-7 decapping activator complex serves as an integration node of regulatory pathways mediating plant tolerance to abiotic stresses. It controls Arabidopsis tolerance to freezing, drought and high salt by interacting with selected, specific and nonspecific, stress-inducible transcripts under each stress condition to promote their decapping and subsequent degradation, which finally ensures the appropriate patterns of downstream stress-responsive gene expression. Interestingly, one of the regulatory pathways mediated by the LSM1-7 complex is the one leading to ABA biosynthesis. We demonstrate that this complex modulates the correct levels of ABA in response to adverse environmental situations through the differential control of *NCED3* and *NCED5* mRNAs turnover.

The expression analysis described in this work revealed that *LSM1-7* genes are differentially regulated in response to abiotic stresses. *LSM1-7* mRNAs accumulate in response to cold but not in response to drought or high salt. The mechanisms underlying this regulation are still under investigation. Consistent with the expression results, an increase in LSM1A and LSM1B protein levels was observed only in plants exposed to low temperature. Remarkably, however, Arabidopsis LSM1 proteins were found to localize to P-bodies in response to the different stresses assayed, indicating that the molecular mechanisms mediating LSM1 localization to these foci do not depend on protein levels. In this way, DCP1 has also been reported to localize to P-bodies under stress conditions even though its levels do not increase (Motomura et al., 2015). These findings suggest that the localization of LSM1 to P-bodies, as that of DCP1 and other Arabidopsis RNA-decay related proteins (Merret et al., 2013; Motomura et al., 2015), is mainly dependent on plant exposure to abiotic stresses. Moreover, since some of these proteins, including LSM1, are required for P-body formation (Xu and Chua, 2009; Perea-Resa et al., 2012), the assembly of these foci should also largely depend on the environmental conditions. How abiotic stresses regulate the cytoplasmic dynamics of proteins involved in RNA degradation and, ultimately, P-body formation remains unknown. Phosphorylation of different components of human and Arabidopsis mRNA decapping machineries by mitogen-activated protein kinases (MPKs) during stress responses is necessary for their cytoplasmic localization and for P-body assembly (Rzeczkowski et al., 2011; Xu and Chua, 2009; 2012; Roux et al., 2015). The sequence of LSM1 proteins (Perea-Resa et al., 2012) contains a consensus motif S/T-P for phosphorylation by MPK. It is, therefore, tempting to speculate that the subcellular localization of LSM1 proteins could be controlled by MPKs in response to abiotic stresses.

Our results demonstrate that the LSM1-7 complex differentially regulates Arabidopsis tolerance to challenging environmental situations. In fact, it restrains its capacity to cold acclimate and tolerate drought while promoting its tolerance to high salt. Several proteins involved in 5'-3' mRNA degradation from plants (AtCAF1a, DCP1, DCP5, XNR4), yeast (CCR4, XRN1) and animals (XRN1, PARN1, PAN1) have been implicated in abiotic stress tolerance, but each of them was related to a particular stress condition (Walley et al., 2010; Castells-Roca et al., 2011; Xu and Chua, 2012; Merret et al., 2013; Nousch et al., 2013; Watanabe et al., 2013). To date, however, the implication of a single component of the mRNA decay machinery in different abiotic stress responses has not been shown in any system, and uncovers the enormous potential of this machinery to precisely modulate the adaptation of a given organism to its surroundings. The LSM cytoplasmic complex, therefore, seems to serve as a regulatory node where pathways mediating abiotic stress responses converge and integrate to guarantee the precise development of Arabidopsis tolerance to freezing, drought and salinity. Our results, furthermore, provide evidence that the LSM1-7 complex operates in Arabidopsis tolerance to abiotic stresses by controlling stress-responsive gene expression. Hundreds of genes are specifically regulated by the complex under low temperature, water deficiency or high salt. Moreover, the complex also modulates the expression of many genes in response to more than one abiotic stress, which indicates that it controls gene expression under different unfavorable situations via specific and shared signaling pathways. Interestingly, numerous specific and nonspecific genes that are regulated by the LSM cytoplasmic complex under each stress condition have been described to have a role in plant tolerance to such condition, substantiating a major and differential function for the complex in plant tolerance to abiotic stresses by ensuring appropriate patterns of stress-responsive gene expression. DCP5 has also been shown to act in abiotic stress tolerance through the regulation of stress-related gene expression. It enhances Arabidopsis tolerance to dehydration by modulating numerous dehydration-responsive genes (Xu and Chua, 2012). Whether DCP5 and/or other components of the Arabidopsis mRNA decay apparatus have the ability, as the LSM1-7 complex, to differentially regulate gene expression in a stress-dependent manner would have to be studied.

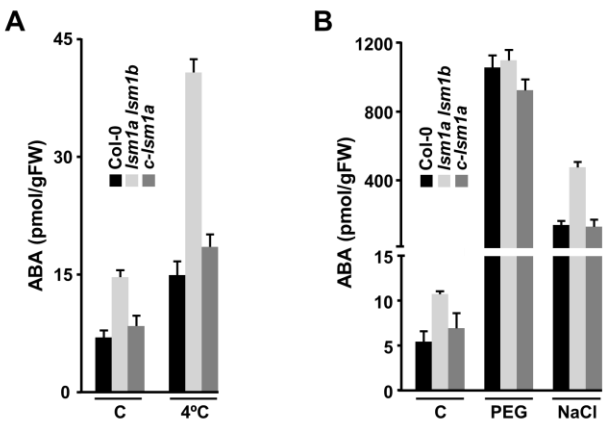

Figure 6. ABA biosynthesis is differentially regulated by the Arabidopsis LSM1-7 complex in response to abiotic stresses.

(A-B) ABA levels in 2-week-old Col-0, *lsm1a lsm1b* and *c-lsm1a* plants grown under control conditions (C), exposed 24h to 4ºC **(A)**, 10h to 55% PEG **(B)** or 10h to 150mM NaCl **(B)**. Error bars indicate SD.

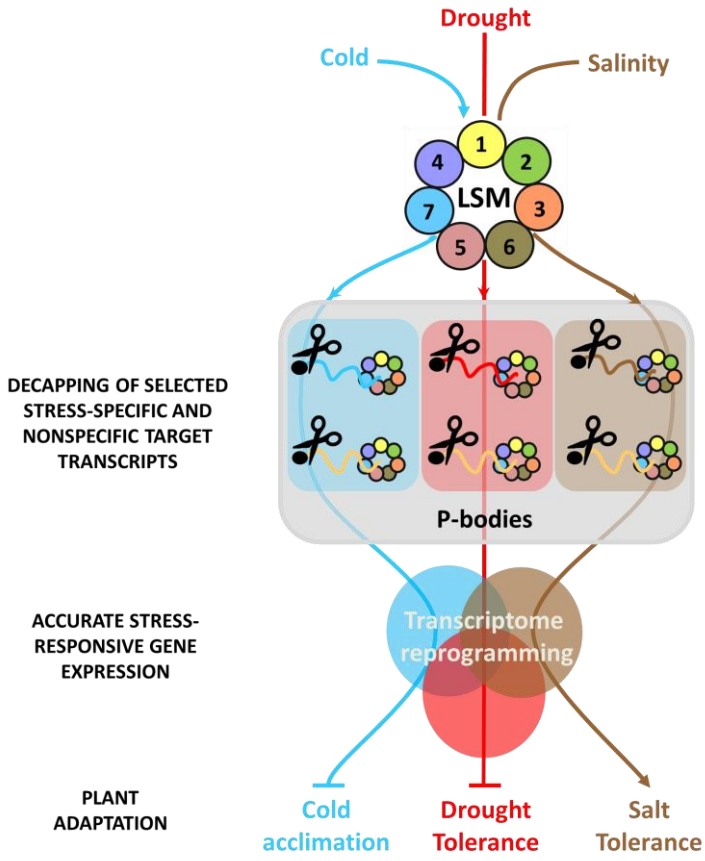

Figure 7. Proposed model for the function of LSM1-7 complex in plant response to abiotic stresses. Arrowheads and end lines indicate positive and negative regulation, respectively.

The role of the LSM cytoplasmic complex in the transcriptome reprogramming that takes place in Arabidopsis when subjected to abiotic stresses is a consequence of its capacity to regulate the turnover of stress-selected target transcripts. In fact, analyzing the interaction between mRNAs whose levels are attenuated by the complex in response to abiotic stresses and the LSM1 protein, we have been able to identify transcripts that are direct targets of the complex when Arabidopsis plants are exposed to low temperature, water stress or high salt. Stress-selected targets are then triggered for decapping and subsequent degradation. It is noteworthy that some of them are specific for each stress while others, however, are selected by the complex in response to various adverse conditions. Modulating the precise half-life of the selected specific and nonspecific target transcripts, therefore, ensures adequate stress-responsive gene expression under each abiotic stress, and ultimately contributes to the capacity of the LSM1-7 complex to regulate Arabidopsis tolerance to freezing, drought and salinity. The aforementioned CCR4, XRN1, PARN1, PAN1, XRN1, DCP5 and XRN4 proteins, belonging to the 5'-3' mRNA degradation machineries from different organisms, have been reported to select target transcripts in response to an individual abiotic stress each (Castells-Roca et al., 2011; Xu and Chua, 2012; Merret et al., 2013; Nousch et al., 2013; Watanabe et al., 2013). Our results, nonetheless, unveil a more intricate scenario when considering the regulation of gene expression and plant adaptation by this post-transcriptional machinery under unfavorable circumstances. They constitute the first evidence so far that one component can select different targets depending on the stress situations, highlighting a new regulatory mechanism of mRNA turnover during stress responses.

Remarkably, among the stress-dependent selected transcripts that are triggered by the Arabidopsis LSM cytoplasmic complex for decapping in response to abiotic stresses, we found those encoding NCED3 and NCED5, two key enzymes in ABA biosynthesis (Tan et al., 2003; Frey et al., 2012). *NCED3* and *NCED5* messengers are direct targets of the complex under low temperature but not under drought conditions. Under salt stress only *NCED5* is targeted by the LSM1-7 complex. ABA is synthesized *de novo* in plants subjected to cold, drought or salinity, and plays a pivotal role in plant responses to abiotic stresses (Finkelstein, 2013). In Arabidopsis, the accumulation of ABA after abiotic stress exposure seems to be the result of *NCED3* and *NCED5* expression (Tan et al., 2003; Frey et al., 2012). So far, available data indicated that this expression is regulated at the transcriptional level (Jiang et al., 2012; Yang and Tan, 2014). The results reported here demonstrate that the mRNA decay machinery, and in particular the decapping apparatus, has a crucial function in establishing the levels of *NCED3* and *NCED5* mRNAs when plants are exposed to stressful circumstances, evidencing that these levels are also controlled by post-transcriptional mechanisms. Our findings, therefore, unveil the existence of a new layer of regulation of ABA biosynthesis in response to abiotic stresses. On the other hand, consistent with its role in differentially modulating the turnover of *NCED3* and *NCED5* transcripts under adverse environmental

circumstances, our data also demonstrate that the LSM cytoplasmic complex contributes to guarantee the correct levels of ABA under cold and high salt conditions. It is not involved, however, in controlling the accumulation of ABA caused by water deficiency.

Based on the results described in this work, a hypothetical model for the function of the Arabidopsis LSM1-7 complex in plant adaptation to abiotic stresses is presented in Figure 7. In response to low temperature, drought and salinity, the complex would preferentially localize to P-bodies. There, depending on the stress, the complex would interact with selected transcripts promoting their decapping and subsequent 5'-3' degradation. A number of selected transcripts are stress-specific, others, however, interact with the complex under more than one stress situation. In most cases, target transcripts would correspond to stress-inducible genes and some of them, moreover, encode proteins involved in regulating, positively or negatively, Arabidopsis tolerance to abiotic stresses. The degradation of the LSM1-7 target transcripts selected in response to a given abiotic stress would have, in turn, a substantial impact on downstream stress-regulated gene expression that would contribute to shape the adequate transcriptome reprogramming required for plant adaptation to that stress. In conclusion, the results presented here reveal that the LSM1-7 decapping activator complex plays a critical role in plant adaptation to abiotic stresses by controlling the turnover of selected stress-specific and nonspecific target transcripts, and, consequently, stress-regulated gene expression. Identifying the molecular mechanisms whereby the LSM cytoplasmic complex chooses different targets depending on the stress conditions is a clear goal of future studies.

EXPERIMENTAL PROCEDURES

Plant materials, growth conditions, tolerance assays and treatments

Arabidopsis Columbia (Col-0) ecotype was used in all experiments. *lsm1a lsm1b* double mutants, transgenic lines *LSM1A$_{PRO}$-LSM1A-GFP* (*c-lsm1a*) and *LSM1B$_{PRO}$-LSM1B-GFP* (*c-lsm1b*), and transgenic lines *c-lsm1a* and *c-lsm1b* expressing the *35S-DCP1-RFP* construct were previously described (Perea-Resa et al., 2012). The *Arabidopsis* transgenic line expressing the *LSMGRP7$_{PRO}$-GRP7-GFP* fusion (Streitner et al., 2012) was kindly provided by Dorothee Staiger (University of Bielefeld, Germany). Tolerance to freezing temperatures was determined on 2-week-old plants grown on soil as described (Catalá et al., 2014). *In vitro* tolerance to water (25% PEG) and salt (150mM NaCl) stresses was assayed on 7-day-old seedlings grown on GM medium as reported (Verslues et al., 2006). The tolerance of 2-week-old plants growing on soil to these stresses was estimated as the surviving individuals after 10 days of water deprivation and 5 days of re-watering, or after watering with 250mM NaCl for 10 days. In all cases, data reported are expressed as means of three independent experiments with 50 plants each. For detailed information on grown conditions and treatments see Supplemental Information.

Microscopic analysis

Subcellular localization of LSM1A-GFP, LSM1B-GFP and RFP-DCP1 fusion proteins was performed by confocal microscopy in roots from 6-day-old transgenic seedlings grown under control or stress conditions. For additional details see Supplemental Information.

ABA measurements

ABA levels were determined in 20mg of 2-week-old WT, *lsm1a lsm1b* and *c-lsm1a* plants grown under control or stress conditions as described (Turečková et al., 2009). All experiments were repeated as four biological replicates employing 10pmol of stable isotope-labelled standard to validate the LC-MS method.

Immunoblot analysis

Total protein was extracted from 2-week-old Arabidopsis *LSM1A$_{PRO}$-LSM1A-GFP*, *LSM1B$_{PRO}$-LSM1B-GFP* and WT plants grown under control or stress conditions as reported (Catalá et al., 2014). Monoclonal anti-GFP (ab290, Abcam) was used as primary antibody, and horseradish peroxidase-conjugated anti-rabbit as secondary antibody. All assays were realized in triplicate employing three independent protein samples.

Gene expression analysis and RNAseq experiments

For gene expression, qPCR experiments were performed as published (Catalá et al., 2014). Primers used are listed in Table S13. All reactions were realized in triplicate employing three independent RNA samples.

For RNAseq experiments, total RNA was extracted from 2-week-old WT, *lsm1a lsm1b* and *c-lsm1a* plants grown under control or stress conditions. See Supplemental Information for additional details. Data from RNAseq experiments have been deposited in the Gene Expression Omnibus database under accession numbers GSE.

Capped mRNA analysis and RIP experiments

Capped transcript levels were determined as described (Perea-Resa et al., 2012) using total RNA from 2-week-old WT, *lsm1a lsm1b* and *c-lsm1a* plants grown under control or stress conditions. For additional details, see Supplemental Information.

In vivo RIP experiments were carried out as reported (Streitner et al., 2012) in 2-week-old *c-lsm1a* and *GRP7-GFP* plants grown under control or stress conditions. For additional details, see Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental information includes seven figures, fourteen tables and supplemental procedures and can be found at https://www.dropbox.com/sh/d46pi305pxcpv0g/AAByFzP4mm5B_QjPr0NU3_cMa?dl=0

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Chapter 8

Identification of *Arabidopsis* Mutants with Altered Freezing Tolerance

Carlos Perea-Resa and Julio Salinas

Abstract

Low temperature is an important determinant in the configuration of natural plant communities and defines the range of distribution and growth of important crops. Some plants, including *Arabidopsis*, have evolved sophisticated adaptive mechanisms to tolerate low and freezing temperatures. Central to this adaptation is the process of cold acclimation. By means of this process, many plants from temperate regions are able to develop or increase their freezing tolerance in response to low, nonfreezing temperatures. The identification and characterization of factors involved in freezing tolerance are crucial to understand the molecular mechanisms underlying the cold acclimation response and have a potential interest to improve crop tolerance to freezing temperatures. Many genes implicated in cold acclimation have been identified in numerous plant species by using molecular approaches followed by reverse genetic analysis. Remarkably, however, direct genetic analyses have not been conveniently exploited in their capacity for identifying genes with pivotal roles in that adaptive response. In this chapter, we describe a protocol for evaluating the freezing tolerance of both non-acclimated and cold-acclimated *Arabidopsis* plants. This protocol allows the accurate and simple screening of mutant collections for the identification of novel factors involved in freezing tolerance and cold acclimation.

Key words Low temperature, Mutant screening, Freezing-tolerant mutants, Freezing-sensitive mutants, Cold acclimation, Constitutive freezing tolerance, *Arabidopsis*

1 Introduction

Plants are sessile organisms continuously adapting to the environmental changes to ensure an appropriate development. Low temperatures are one of the most important environmental constraints that limit the development and survival of plants and determine their geographical distribution [1]. The stress induced by low temperatures also produces important economic losses, reducing the yield of agricultural crops every year. It is known that modest increases in the freezing tolerance of crop species would positively affect agricultural production [2]. Plants from temperate regions have evolved an adaptive response, known as a cold acclimation [1, 3], whereby they develop or increase their freezing tolerance after being

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exposed during several days to low, nonfreezing temperatures (0–10 °C). Understanding the molecular mechanisms underlying this response is essential to conceive how plants grow and develop under adverse conditions originated by abiotic stresses and to generate new biotechnological strategies to improve crop tolerance to freezing temperatures and other related stresses such as drought and high salt.

Genetic analysis is a classical and powerful tool for identifying genes implicated in a given physiological process. In the case of freezing tolerance, the identification and characterization of mutant plants with altered freezing tolerance before and/or after cold acclimation have been carried out essentially in *Arabidopsis*, a model plant that is able to acclimate to low temperature increasing its constitutive freezing tolerance. Its small genome, the first to be sequenced in plants, together with its physiological characteristics, facilitates the subsequent molecular identification and characterization of the mutated genes. The most commonly used mutants in the screenings were generated by *e*thyl *m*ethane*s*ulfonate (EMS), an organic compound that randomly produces nucleotide substitutions in the DNA [4–6]. For instance, Warren et al. [7] identified several *Arabidopsis* EMS mutants, termed *sensitivity to freezing* (*sfr*), that showed reduced freezing tolerance compared with wild-type (WT) plants. Consistent with the expectations that *sfr* should be loss-of-function mutations, most of them were recessive. Seven *sfr* mutants were nonallelic and only acquired partial freezing tolerance after cold acclimation. A preliminary study revealed that four *sfr* mutations, *sfr3*, *sfr*4, *sfr*6, and *sfr7*, reduced or blocked anthocyanin accumulation during this adaptive response. *sfr4* mutant was also impaired in cold-induced accumulation of sucrose and glucose levels, and both *sfr4* and *sfr7* mutants showed abnormal fatty acid composition when cold acclimated [8]. In another study [9], Xin and Browse identified several *Arabidopsis* EMS mutants with increased freezing tolerance. One of them, *eskimo1 (esk1)*, presented an increase in both constitutive freezing tolerance and cold acclimation capacity. *esk1* was originated by a single recessive mutation in the *AT3G55990* locus that produced elevated proline levels but did not generate constitutive expression of cold-regulated genes. Finally, Llorente and colleagues [10] identified *freezing sensitive 1* (*frs1*), an *Arabidopsis* EMS mutant that exhibited decreased constitutive freezing tolerance and capacity to cold acclimate. Complementation analysis revealed that *frs1* mutation was a new allele of *ABA3*, supporting that ABA is essential for full development of cold acclimation and for constitutive freezing tolerance in *Arabidopsis*.

In this chapter, we describe a simple and precise protocol for evaluating the freezing tolerance of both non-acclimated and cold-acclimated *Arabidopsis* plants. The protocol is suitable for screening mutants generated by EMS, fast neutron (FN), or T-DNA

insertions and can be carried out with plants grown on media, in Petri dishes, or on soil, in pots. Important aspects, depending on searching for mutants with increased (tolerant) or decreased (sensitive) freezing tolerance, are also detailed. In addition, this protocol can also be used in reverse genetic studies to determine the involvement of a gene of interest in freezing tolerance and to assess the effect that different treatments may produce on the tolerance of *Arabidopsis* to freezing temperatures.

2 Materials

2.1 Plant Material

WT seeds of the appropriate ecotype, mutagenized M_2 families or M_2 pools, depending if screening for sensitive or tolerant mutants, respectively (*see* **Note 1**), and seeds of previously reported tolerant and/or sensitive freezing mutants to be used as controls in the screenings [7, 9].

2.2 Plate Assay

1. 1.5 mL Eppendorf or 50 mL Falcon tubes.
2. MS growth media (0.5× Murashige and Skoog basal salt mix; 2.5 mM *m*orpholino *e*thane*s*ulfonic acid (MES), pH 5.7; 0.8 % agar).
3. Amphotericin B (final concentration 2.5 mg/L).
4. Round plates (Ø 15 cm).
5. 3 M Micropore tape.
6. Filter paper or nylon mesh.
7. Bell jar.
8. Bleach.
9. HCl.
10. Forceps.
11. Liquid nitrogen.
12. Mortar and pestle.
13. Spoon.

2.3 Soil Assay

1. Peat substrate.
2. Vermiculite.
3. Clay pots (Ø 10 cm).
4. Trays.
5. Plastic film.

2.4 Growth Chambers and Other Equipment

1. Plant growth chamber set at 20–22 °C with cool-white light (100 μE/m²/s).
2. Plant growth chamber set at 4 °C with cool-white light (50 μE/m²/s).

3. Plant growth chamber with a range of programmable temperatures from 4 °C to −14 °C with lights off.
4. Cold room.
5. Autoclave.
6. Fume hood.
7. Sterile hood.

3 Methods

3.1 Seed Mutagenesis

Protocols to obtain EMS or FN mutagenized seeds have been previously described [5, 11]. T-DNA mutant collections can be generated as reported [12]. EMS and FN mutagenized seeds from different ecotypes are also commercially available at Lehle seeds (http://www.arabidopsis.com), while T-DNA mutant collections can be ordered at the European Arabidopsis Stock Centre (NASC). Generation of M_2 families and M_2 pools from M_1 mutagenized seeds has already been communicated [4] (*see* **Note 1**).

3.2 Screening Using Plates

We strongly recommend vapor-phase seed sterilization using chlorine gas (*see* **Note 2**).

3.2.1 Seed Sterilization and Plating

1. Put WT, control, and mutagenized seeds into appropriate tubes depending on number (Eppendorf or Falcon tubes are suitable).
2. Open the tubes into a hermetic bell jar placed in a fume hood.
3. Generate chlorine gas by combining 100 mL of bleach and 3 mL of HCl in a 200 mL glass placed inside the jar.
4. Close the jar, and let the seeds be exposed to chlorine gas for 3 h.
5. Open the jar inside the fume hood, and air-ventilate the seeds for 15 min before closing the tubes.
6. Cut filter paper pieces according to the plate size, and autoclave (*see* **Note 3**).
7. Place sterile filter papers on MS plates by using sterilized forceps.
8. Distribute sterilized seeds over the sterile papers (*see* **Note 4**).
9. For seed stratification seal plates with 3M Micropore tape and transfer them to a cold room (4 °C) under darkness for 2 days.
10. Transfer plates to a growth chamber, and let seeds germinate and develop for 12 days at 20–22 °C under long-day conditions (16-h light/8-h darkness).

3.2.2 *Freezing Assay*

1. Transfer plates to the growth chamber for freezing assay (*see* **Notes 5** and **6**), and expose seedlings to freezing temperatures under dark conditions. Appropriate temperatures should be empirically established depending on the accessions employed, the type of seedlings used in the screening (non-acclimated or cold-acclimated), and the searched mutants (tolerant or sensitive to freezing) (*see* **Notes 7** and **8**) (Fig. 1).
2. Prepare fresh ice chips by vaporizing sterile distilled water in a mortar containing liquid nitrogen. Grind the ice to a fine powder, and gently apply over the seedlings homogeneously when temperature decreases to –2 °C. Close the plates, and let the program finish (*see* **Note 9**).
3. After the freezing assay, when seedlings are exposed to 4 °C and media is still frozen, move plates to a sterile hood and,

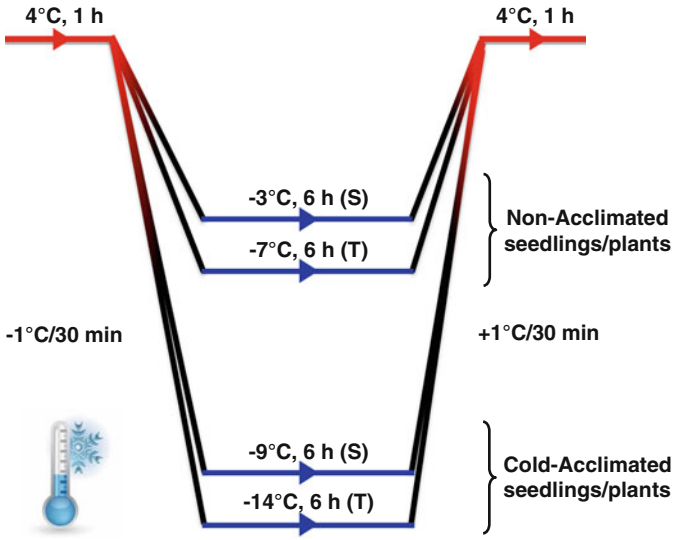

Fig. 1 Schematic representation of the freezing program used for the screenings. In all cases, before being subjected to freezing temperatures, seedlings and plants are exposed for 1 h to 4 °C in the freezing chamber. Then, temperature is progressively decreased (–1 °C/30 min) until reaching the desired freezing temperature. As an example, the two different temperatures we generally use to screen for non-acclimated seedlings or plants (–3 and –7 °C) and the two temperatures we generally use to screen for cold-acclimated seedlings or plants (–9 and –14 °C) are shown. –3 and –9 °C are employed when looking for sensitive mutants (S), while –7 and –14 °C when looking for tolerant ones (T). After exposing plants to the appropriate freezing temperature for 6 h, temperature is gradually increased to 4 °C (+1 °C/30 min). One hour later, plants are transferred to 20 °C under long-day light regime for recovering and subsequent survival evaluation

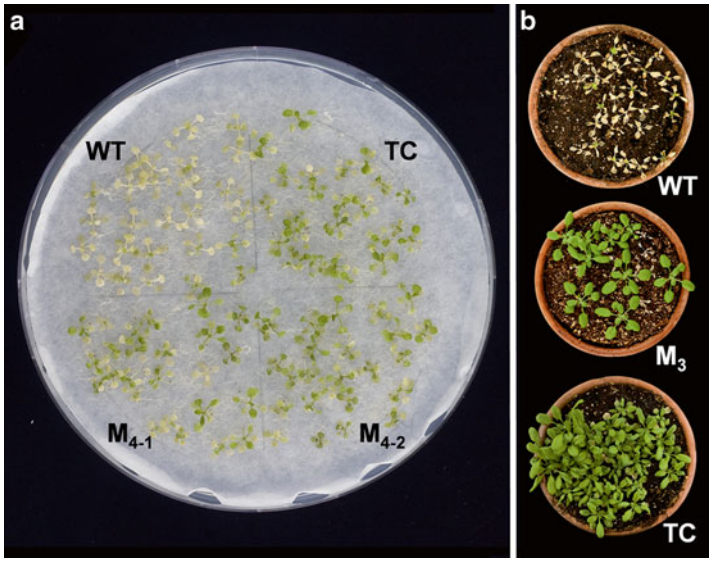

Fig. 2 Identification of *Arabidopsis* mutants with increased freezing tolerance before and after cold acclimation. (**a**) Non-acclimated seedlings 1 week after being frozen on plate at −7 °C for 6 h. Col-0 (WT), tolerant control (TC), and two M_4 homozygous families for two tolerant mutations are shown. (**b**) Cold acclimated plants 1 week after being frozen on pot at −14 °C for 6 h. Col-0 (WT), tolerant control (TC), and an M_3 family segregating for a tolerant mutation are shown

using sterilized forceps, transfer carefully the filter papers with the frozen seedlings to new plates containing MS media supplemented with amphotericin B (*see* **Notes 10** and **11**).

4. Move seedlings to a growth chamber, and let them to recover at 20–22 °C for 1 week under long-day light regime. An example of recovered seedlings is shown in Fig. 2a (*see* **Note 12**).
5. When screening for tolerant mutants, transfer surviving M_2 seedlings to soil and allow them to reproduce for phenotype confirmation in a secondary screening with the corresponding M_3 families (*see* Subheading 3.4).
6. If screening for sensitive mutants, repeat the freezing assays with additional seeds from the selected M_2 lines (*see* **Note 13**), confirm the survival rate, and proceed to phenotype confirmation (*see* Subheading 3.4).

3.3 Freezing Using Pots

3.3.1 Pot Preparation and Growing Conditions

1. Mix peat substrate with vermiculite in a 3:1 ratio, add one volume of water per 3 volumes of mix, and sterilize at 120 °C for 20 min.
2. Fill pots homogeneously with sterile soil avoiding leaving air bubbles (*see* **Note 14**).
3. Distribute the seeds on the soil as separated as possible (*see* **Note 15**).

4. Place the pots into trays, cover it with plastic film for humidity maintenance during the first days after germination, and transfer them to a cold room (4 °C) under darkness for 2 days for stratification.
5. Move the trays containing the pots to a growth chamber set at 20–22 °C with a long-day light regime, and allow seeds to germinate and develop for 2 weeks.

3.3.2 Freezing Assay

1. Transfer individual pots to the growth chamber for freezing assay (*see* **Notes 6** and **16**), and expose plants to freezing temperatures.
2. Screening conditions are essentially the same as those described for seedlings on plates (*see* **Notes 7**, **8**, and **17**) (Fig. 1).
3. After the freezing assay, move plants to a growth chamber and let them to recover at 20–22 °C for 1 week under long-day light regime (Fig. 2b).
4. When screening for tolerant mutants, allow surviving M_2 plants to reproduce for phenotype confirmation in a secondary screening with the corresponding M_3 families (*see* below).
5. If screening for sensitive mutants, repeat the freezing assays with additional seeds from the selected M_2 lines (*see* **Note 13**), confirm the survival rate, and proceed to phenotype confirmation in a secondary screening (*see* below).

3.4 Confirmation of Mutant Phenotypes

1. When screening for freezing-tolerant mutants, screen about 100 seedlings or plants from each generated M_3 family for their freezing tolerance, and calculate the survival rate.
2. M_3 families from freezing tolerant M_2 seedlings or plants showing survival rates of 100 % are likely produced by a single mutation in homozygosis. Families showing a 3:1 or 1:3 tolerant/sensitive ratio would be generated by a single dominant or recessive mutation in heterozygosis, respectively (*see* **Note 18**). In these cases, select and reproduce at least ten freezing-tolerant seedlings or plants to obtain the corresponding M_4 families. The screening of these will allow the identification of the homozygous mutant lines (*see* **Note 19**).
3. If screening for freezing-sensitive mutants, collect seeds from at least ten M_2 plants from each selected M_2 family to obtain the corresponding M_3 ones.
4. The screening of around 100 seedlings or plants from each generated M_3 family will allow the identification of homozygous mutant lines for the phenotype selected (those showing survival rates of 0 %).

3.5 Molecular Identification of Selected Mutations

The number of mutations that a selected homozygous mutant line contains in its genome varies depending on the method employed for seed mutagenesis. Before proceeding to the molecular identification

of the mutations, mutant lines should be backcrossed several times in order to clean up their genetic backgrounds of mutations not related to the freezing phenotype of interest.

Until 2 or 3 years ago, the molecular identification of EMS- or FN-produced mutations causing a phenotype of interest in *Arabidopsis* was performed through a map-based cloning approach. This method is based on crossing a mutant plant of interest with a WT of a different accession. Subsequent polymorphism analysis determines the chromosome region where the mutation is located and, finally, by complementation experiments, the mutagenized locus is identified [13]. Unfortunately, the enormous amount of time required to identify a mutation causing a phenotype of interest by this method constitutes an important obstacle when planning a forward genetic screening. During the last 2 years, next-generation genomic sequencing technologies have been applied for the rapid and precise molecular identification of mutations in different *Arabidopsis* ecotypes. For instance, Austin and colleagues [14] have described a rapid and robust method for mapping mutations, independently of their chromosomal location, by sequencing only a small pooled M_2 population. The protocol was able to identify a highly restricted region containing very few SNP candidates that can be easily validated using standard reverse genetic techniques. On the other hand, Uchida et al. [15] have been able to identify a mutated locus in a non-reference *Arabidopsis* accession, i.e., whose genome is not publicly available, by only one round of genome sequencing.

When screening mutant collections generated by T-DNA insertions, identifying the site of insertion in the genome is commonly performed using an adapter ligation-mediated PCR protocol [16]. This method consists of three steps and takes about 3 weeks to be completed. First, an adapter is ligated to genomic DNA after digestion with a restriction enzyme. Then, by using specific primers to the adapter and T-DNA, the T-DNA/genomic DNA junction is amplified by PCR. Finally, sequencing the T-DNA/genomic junction allows mapping the T-DNA location in the genome.

4 Notes

1. M_2 families collected by pedigreeing are the recommended material to screen for sensitive mutants. Since sensitive mutants will not survive the screening, you must ensure a high (>200) number of mutant seeds for each family. When screening for tolerant mutants, M_2 pools are the material of choice. Generation of M_2 families and M_2 pools has been reported in detail [4].

2. Seeds can also be sterilized with bleach as described [17].
3. Wrap paper pieces with aluminum paper. For a correct sterilization, do not autoclave more than ten paper pieces together. Other supports, such as a nylon mesh, can also be used.
4. The seed number per plate depends on the germination rate and on the type of screening that is going to be performed. When screening for freezing-tolerant mutants, a high number of pooled M_2 seeds can be plated (~300 seeds/Ø15 cm plate). When the screening is performed to identify freezing-sensitive mutants, the number of seeds plated from each M_2 family should allow establishing a significant sensitive/tolerant segregation (~100 seeds/Ø15 cm plate).
5. For evaluation of freezing tolerance after cold acclimation, before freezing, plates should be transferred to a growth chamber set at 4 °C for 5 days under long-day conditions to ensure the full development of the adaptive response.
6. We strongly recommend transferring seedlings or plants to the appropriate growth chamber for the freezing assay always at the same time of the day, since the expression of several cold-regulated genes involved in cold acclimation is subjected to circadian regulation [18, 19].
7. Appropriate freezing temperatures to evaluate the tolerance of non-acclimated or cold-acclimated seedlings depend on the type of screening to be performed (searching for freezing-tolerant or -sensitive mutants) and should be previously established in each case using WT seedlings and previously reported tolerant and/or sensitive mutants that will act as positive controls. If searching freezing-tolerant mutants, the highest temperature that produces 0 % surviving seedlings should be used. On the contrary, when screening for freezing-sensitive mutants, the lowest temperature that allows 100 % seedling survival is the convenient one. The time that seedlings should be exposed to the appropriate freezing temperatures must be determined at the same time as freezing temperatures. Different temperatures are used for the evaluation of non-acclimated and cold-acclimated seedlings, the latter always requiring lower temperatures (~2–3 °C). Optimally, freezing temperatures must be gradually reached (−1 °C/30 min) starting from 4 °C (Fig. 1).
8. During the freezing assay, it is critical that the temperature inside the chamber is homogeneous in such a way that all seedlings being screened are exposed to the same conditions.
9. Ice chips are ice nucleation sites that favor freezing homogeneity in all seedlings of the plate.
10. Seedlings should be transferred to new plates since freezing temperatures depolymerize the growth media.

11. Amphotericin B is a polyene antifungal drug that helps to minimize plate contaminations.
12. In our experience, 1 week of recovery is enough to establish if seedlings have survived to the freezing treatment or not. Fungal contaminations usually appear when longer recovering times are allowed.
13. We recommend selecting M_2 lines with a 3:1 or a 1:3 sensitive/tolerant segregation, indicating that the sensitive phenotype is produced by a single dominant or recessive mutation, respectively. Phenotypes produced by single mutations are preferred because these mutations are easy to map and molecularly identify.
14. In our hands, clay pots allow water transpiration and work better than plastic pots.
15. The seed number per pot depends on the germination rate and on the type of screening that is going to be performed. When screening for freezing-tolerant mutants, a high number of pooled M_2 seeds can be sown (~60 seeds/Ø 10 cm pot). When the screening is performed to identify freezing-sensitive mutants, the number of seeds sown from each M_2 family should allow to establish a significant sensitive/tolerant segregation (~40 seeds/Ø 10 cm pot).
16. When screening for mutants with altered freezing tolerance after cold acclimation, plants should be previously exposed to 4 °C during 1 week to ensure the adaptive response.
17. When freezing plants grown on soil, the addition of ice chips is not necessary because freezing occurs very homogeneously on the surface of the pot.
18. Other segregations will suggest that the freezing mutant phenotype is originated by more than one mutation.
19. When screening for freezing-tolerant mutants by pooling, mutant seedlings or plants containing the same mutation may be selected. Allelism tests should then be performed between the identified mutant lines.

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4. DISCUSIÓN

Las proteínas LSM han sido identificadas y caracterizadas en diversas especies procariotas y eucariotas, revelando su importancia funcional. Su existencia en plantas, sin embargo, no ha sido aún demostrada. El estudio llevado a cabo en la presente tesis doctoral establece la conservación del complejo citoplásmico LSM1-7 en *Arabidopsis thaliana* L., y describe su función en el desarrollo y respuesta a estrés de esta especie regulando la expresión génica a nivel post-transcripcional.

Estudios filogenéticos proponen un modelo según el cual, a partir de un único antecesor con un motivo Sm, aparecieron el resto de componentes de la familia SM tras fenómenos de duplicación y divergencia (1). Al igual que en levaduras y humanos, en Arabidopsis existen ocho proteínas LSM altamente conservadas que originan dos complejos heteroheptaméricos con forma de anillo, LSM1-7 y LSM2-8, una estructura que garantiza la interacción con el RNA (2) y, en consecuencia, facilita la participación de estas proteínas en diversos aspectos de su metabolismo. La función y localización de estos complejos LSM está definida por las proteínas LSM1 y LSM8, subunidades que en levaduras compiten por la unión y localización del resto de componentes del complejo en el citoplasma o núcleo, respectivamente (3). La localización específicamente citoplásmica de LSM1 y nuclear de LSM8, junto con su capacidad de interacción con otras LSMs, evidenció un patrón de organización similar en Arabidopsis. Asimismo, la caracterización funcional de estas dos subunidades demostró su papel clave en la formación de los respectivos complejos citoplásmico y nuclear, sugiriendo la existencia de un modelo de competición similar al propuesto en levaduras. Curiosamente, plantas con ausencia de función de las proteínas LSM1 o LSM8 resultaron ser viables. Sin embargo, la ausencia de ambas subunidades, o de alguno de los componentes comunes (LSM2-7), resultó ser letal, indicando la necesidad de la existencia de al menos un complejo LSM para la viabilidad de Arabidopsis.

El extremo 5´cap constituye una estructura esencial para la traducción de los mensajeros. Además, supone un elemento protector, junto con la cola de poli (A), frente a la digestión mediada por enzimas exonucleasas. Resultan clave, por tanto, los mecanismos moleculares que modulan la dinámica de formación y de eliminación (*decapping*) de esta estructura. El proceso de *decapping* es llevado a cabo fundamentalmente en los *P-bodies* por el complejo formado por las proteínas DCP1 y DCP2 (4). Además, otros componentes que participan en la regulación de DCP2 han sido identificados, entre los que destaca, con una función activadora, el complejo LSM1-7 (5). La localización del complejo LSM1-7 de Arabidopsis en *P-bodies* en respuesta a situaciones de estrés, sugería su participación en el proceso de *decapping*. Los ensayos de inmunoprecipitación de RNA, junto con la cuantificación de mensajeros capeados en plantas mutantes *lsm1a lsm1b*, establecieron que, efectivamente, el complejo LSM1-7 funciona como activador del *decapping* mediante su unión directa a mRNAs. La identificación de mensajeros diana del complejo demostró su participación en el control directo de la expresión de genes involucrados en distintos aspectos del desarrollo de Arabidopsis, así como en su respuesta a diferentes estreses de origen abiótico. Curiosamente, algunos de los blancos identificados en plantas de dos semanas no lo son en el estadio de plántulas de seis días, lo que demuestra que el control mediado por LSM1-7 es dependiente de la fase de desarrollo. Además, el análisis comparativo de mRNAs diana del complejo en condiciones de frío, sequía y salinidad, reveló que algunos mensajeros son específicos de cada estrés mientras otros son blancos del complejo en respuesta a varios estreses. En cualquier caso, estos tránscritos diana participan en diversas vías de señalización relacionadas con la tolerancia a situaciones adversas, lo que demuestra la versatilidad del complejo LSM1-7 para regular la respuesta a estrés de Arabidopsis. Merece destacar que entre ellos identificamos los mRNAs que codifican NCED3 y NCED5, dos enzimas clave en la síntesis *de novo* del ácido abcísico (ABA) ante condiciones desfavorables (6-8). El ABA juega un papel fundamental en el desarrollo de la tolerancia de las plantas a estreses abióticos y, en consecuencia, su metabolismo está regulado de manera muy precisa (9-12). Nuestros resultados revelaron que el complejo LSM1-7 promueve la degradación de los tránscritos *NCED3* y *NCED5* en respuesta a frío, mientras que en condiciones de estrés salino únicamente *NCED5* es diana del complejo. Ante situaciones de sequía, por el contrario, ninguno de estos mRNAs es regulado por LSM1-7. Como era de esperar, las medidas de ABA realizadas en plantas *lsm1a lsm1b* sometidas a frío, sequía o altas concentraciones de sal, indicaron que LSM1-7 controla los niveles de esta hormona en respuesta a temperaturas bajas y salinidad, pero no ante situaciones de estrés hídrico. El complejo, por tanto, controla de manera diferencial la expresión de los genes *NCED3* y *NCED5* a nivel post-transcripcional, lo que constituye un nuevo nivel de regulación de la síntesis de ABA en respuesta a estrés en Arabidopsis. En definitiva, el complejo LSM1-7 se erige como un componente esencial en el control de la reprogramación génica durante el desarrollo y la tolerancia a estrés abiótico de Arabidopsis promoviendo el *decapping* y la degradación selectiva de mRNAs.

Los resultados presentados en esta tesis demuestran la existencia del complejo LSM1-7 en Arabidopsis, un activador del proceso de *decapping* clave en la vía de degradación 5´-3´-XRN4. Además, han permitido identificar, por vez primera, mensajeros diana de un factor de degradación en respuesta a diferentes situaciones de estrés. Asimismo, el análisis comparativo de tránscritos diana de LSM1-7 pone de manifiesto su control selectivo sobre la expresión génica mediante la unión directa a mRNAs a través de la subunidad LSM1. El descubrimien-

to de los mecanismos moleculares que posibilitan la especificidad mediada por el complejo en respuesta a señales internas y externas, constituye un tema apasionante para futuros estudios.

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5. CONCLUSIONES

1. El complejo citoplásmico LSM1-7 de Arabidopsis controla la expresión génica a nivel post-transcripcional regulando el metabolismo de mensajeros específicos. Esta función está mediada a través de la unión directa de LSM1 con los mRNAs, promoviendo su *decapping* y consiguiente degradación.

2. El complejo LSM1-7 regula el correcto desarrollo de Arabidopsis y su adecuada adaptación a situaciones ambientales adversas controlando la degradación selectiva de mensajeros en función de señales tanto internas como externas.

6.- ANEXO I: ABSTRACT

Post-transcriptional control of *Arabidopsis thaliana* L. development and abiotic stress response mediated by the LSM1-7 decapping activator complex

6.1. Introduction

Plants are sessile and, consequently, extremely dependent on their capacity to modulate gene expression to achieve their correct development and adaptation against adverse environmental conditions. During the last years, several studies have revealed the importance of post-transcriptional regulation of gene expression in plant development and tolerance to environmental stresses (1-3). Controlling mRNA decay appears to be a rapid way to fine-tune transcript levels in a transcriptional-independent manner. It is mainly accomplished following mRNA deadenylation, decapping and subsequent 5´-3´ mRNA degradation (4). This highly regulated process occurs in cytoplasmic discrete foci named Processing Bodies (*P-bodies*), where mRNAs and components of the decay machinery localize and interact (5, 6). Several factors have been described to coordinate the different molecular events that constitute the mRNA degradation process. Among them, SM-like (LSM) proteins seems to play an essential function as RNA chaperones, orchestrating multiple RNA-protein interactions and enzymatic reactions (7-9).

LSMs belong to the SM protein family, whose antibodies were firstly identified in a patient with a form of systemic lupus erythematous (10). The existence of LSM proteins in prokaryotes, bacterial and eukaryotes, reveal their importance. LSMs are organized in two ring-shaped complexes formed by seven different components each, LSM1-7 and LSM2-8, and operate in important aspects of RNA metabolism. LSM1 and LSM8 subunits define each complex, determining its subcellular localization and function. In yeast, LSM2-8 localizes into the nucleus and is involved in splicing through the stabilization of the U6 snRNA, an essential component of the spliceosome (11). The other complex, LSM1-7, localizes in the cytoplasm, concretely to *P-Bodies*, and binds to mRNAs promoting their decapping and subsequent degradation (7). *In-silico* data suggested the existence of plant *LSM* genes (12), however, their molecular and functional characterization remain unknown.

6.2. Objectives

In this PhD work, I aimed at the identification and functional characterization of the Arabidopsis LSM1-7 complex. Furthermore, I studied the role of this complex in Arabidopsis development and adaptation to stress situations. The PhD dissertation includes three publications. The first one, published in The Plant Cell, contains the results regarding the identification and functional characterization of LSM1-7 complex from Arabidopsis. Moreover, in this paper we describe an essential function for LSM1-7 in regulating Arabidopsis development. A second article, currently under revision in Molecular Cell, reports on the function of LSM1-7 complex in plant response to different abiotic stress conditions. Finally, derived from the study of the role of LSM1-7 in cold response, a detailed protocol for evaluating the freezing tolerance of Arabidopsis mutants was developed. This protocol was published as a chapter in the book entitled "Plant Cold Acclimation: Methods and Protocols", belonging to Methods in Molecular Biology series edited by Springer Science, and is also included in this dissertation.

6.3. Results

6.3.1 Identification and functional characterization of the Arabidopsis LSM1-7 complex and its implication in plant development

A total of 11 *LSM* genes were previously annotated in the Arabidopsis genome, three of them, *LSM1*, *LSM3* and *LSM6*, being duplicated (12). Our analysis revealed similar expression patterns for all Arabidopsis *LSM* genes under control conditions. Moreover, all of them are induced in response to cold, but not under drought or high salt conditions. Protein-protein interaction assays demonstrated the physical interaction between Arabidopsis LSMs following a conserved pattern similar to that previously described in yeast. Thus, two different heteroheptameric complexes were identified, LSM1-7 and LSM2-8. LSM2-7 form the core of each complex, while the seventh component, LSM1 or LSM8, determines its subcellular localization and function. LSM1-7 localizes in the cytoplasm, accumulating to *P-bodies* under challenging situations such us low temperature, drought and salinity. Interestingly, the absence of *P-bodies* in plants defective in *LSM1A* and *LSM1B* expression (*lsm1a lsm1b* mutant, see below) revealed an essential role for LSM1-7 in *P-body* formation. Taken together, all these data demonstrated the existence of a LSM1-7 cytoplasmic complex in Arabidopsis defined by the LSM1 subunit.

In an attempt to functionally characterize the LSM1-7 complex, mutant plants containing T-DNA insertions in *LSM1A* or *LSM1B* genes (*lsm1a* and *lsm1b*) were identified. They were indistinguishable from Col-0 wild type (WT) plants. *lsm1a lsm1b* double mutants, however, exhibited important alterations in several organs at different developmental stages ranging from seeds to

flowers. Transformation of *lsm1a lsm1b* with genomic *LSM1A* or *LSM1B* constructs, rescues all mutant phenotypes, revealing redundant functions for LSM1A and LSM1B proteins and, more importantly, an essential role of LSM1-7 in Arabidopsis development.

The LSM1-7 complex was described to modulate gene expression by promoting mRNA decay in yeast (7). To gain insight into the function of Arabidopsis LSM1-7 complex in plant development, we analyzed the impact of *lsm1a* and *lsm1b* mutations on global gene expression by means of microarray experiments. About 700 genes showed de-regulated expression (±2-fold) in two-week-old *lsm1a lsm1b* mutants compared with WT plants grown under standard conditions. Interestingly, an important percentage of these genes (10%) were involved in plant development at different stages, and would account for the diverse developmental alterations observed in double mutant plants. Considering the capacity of the LSM1-7 complex to regulate the turnover of transcripts through the interaction of LSM1 with target mRNAs to promote their decapping and subsequent degradation (7, 9), we reasoned that it could control plant development by promoting mRNA decay. Indeed, mRNA half-life measurements in WT and *lsm1a lsm1b* plants evidenced the role of LSM1-7 in controlling the turnover of selected developmental-related transcripts. It is noteworthy that these mRNAs accumulated in their capped forms in double mutants, demonstrating the function of LSM1-7 in mRNA decay as a decapping activator. Intriguingly, some transcripts, identified as LSM1-7 direct targets in two-week-old plants, were not in 6-day-old seedlings, indicating that the function mediated by LSM1-7 is developmental stage dependent. All in all, our results uncovered a key role for LSM1-7 complex in plant development by controlling gene expression through activating the decapping of developmental-related mRNAs.

6.3.2 Role of the LSM1-7 complex in Arabidopsis tolerance to abiotic stresses

LSM1 transcripts and the corresponding LSM1 proteins accumulate in response to cold. Moreover, under low temperature, drought and high salt conditions, LSM1 localizes to *P-bodies*. All these data strongly suggested a function for Arabidopsis LSM1-7 complex in abiotic stress responses. To test this assumption, the tolerance of *lsm1a lsm1b* plants to freezing temperatures, water stress and high salt was evaluated. *lsm1a lsm1b* plants showed increased freezing tolerance after cold acclimation, the adaptive process whereby many plants augment their tolerance to freezing in response to low temperature (13), indicating a negative role for LSM1-7 in this process. Similarly, *lsm1a lsm1b* double mutants were also more tolerant to drought than WT plants, suggesting also a negative role for LSM1-7 in response to water stress. In contrast, mutant plants showed increased sensitivity to high salt, which indicated a positive role for LSM1-7 in salt tolerance. All these data unveiled a differential and pivotal function for the LSM1-7 complex in plant tolerance to environmental adverse situations.

In an attempt to understand how the LSM1-7 complex differentially regulated Arabidopsis tolerance to abiotic stresses, global transcriptome analysis of WT and *lsm1a lsm1b* plants exposed to low temperature, drought and high salt, were performed by means of RNA sequencing (RNAseq). In all cases, *lsm1a lsm1b* plants showed alterations in the expression of a very high number of genes. A total of 3124 genes presented altered expression (±2-fold) in mutant plants in response to cold, 1999 in response to drought, and 2597 in response to high salt. The difference between these and those numbers obtained when analyzing gene expression in *lsm1a lsm1b* under standard conditions is likely due to the different technologies employed (RNAseq vs microarray). Numerous stress-inducible genes were identified in all cases, the expression of most of them being specifically deregulated in response to one single stress. Importantly, many of these genes had been previously reported to be involved in plant tolerance to freezing, drought and/or salinity, which should account for the tolerance phenotypes displayed by *lsm1a lsm1b* mutants.

To determine how the LSM1-7 complex differentially modulated stress-responsive gene expression, we identified LSM1-7 direct mRNA targets under abiotic stress conditions. *In vivo* RNA inmunoprecipitation (RIP) assays demonstrated the capacity of LSM1-7 to physically interact with selected different transcripts, promoting their decapping and subsequent decay. Remarkably, some of these mRNAs have been described to play positive function on cold acclimation and water stress tolerance. In response to salt stress, various identified target transcripts have been reported to play a negative role in salt tolerance. Our results also indicate that some LSM1-7 targets are stress-specific. However, according to the close relationship existing between plant responses to low temperature, water deficiency and high salt (14, 15), a number of them are targets of the complex in response to more than one stress condition. Interestingly, among the transcripts differentially regulated by LSM1-7 we identified those encoding NCED3 and NCED5, two key enzymes involved in the biosynthesis of abcisic acid (ABA), an essential phytohormone in plant tolerance to abiotic stresses (16-18). While both mRNAs are targets of LSM1-7 in response to low temperatures, none of them are targets in response to drought, and only *NCED5* mRNA is target in response to high salt. As a consequence, LSM1-7 negatively regulates ABA levels in response to cold and high salt, but not in response to drought, revealing a new layer of control of ABA biosyn-

thesis, and uncovered a stress-selective mRNA decay control mediated by LSM1-7 complex in Arabidopsis.

On the whole, our study revealed a novel post-transcriptional mechanism involved in gene expression regulation in plants. This mechanism, based on LSM1-7 binding to mRNA and subsequent decapping activation, is achieved over selected transcripts depending on internal and external signals. Deepen in the understanding of the molecular mechanisms involved in LSM1-7 specificity constitutes a fascinating challenge for future studies.

6.4. Conclusions

1. The Arabidopsis cytoplasmic LSM1-7 complex control gene expression at the post-transcriptional level regulating the turnover of selected mRNAs. This function is mediated by the direct interaction between LSM1 and mRNAs, promoting its decapping and subsequent decay.

2. The LSM1-7 complex ensure the correct development of Arabidopsis and its adequate adaptation to adverse environmental conditions controlling selective mRNA decay depending on internal and external signals.

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7.- ANEXO II: RESUMEN/SUMMARY

RESUMEN

Palabras clave: Arabidopsis, regulación post-transcripcional, *decapping*, expresión génica, desarrollo, estrés abiótico

La regulación de la expresión génica juega un papel clave en los procesos de diferenciación celular a lo largo del desarrollo de los organismos vivos. Además, la reprogramación de dicha expresión es esencial para su correcta adaptación al medio que les rodea. Las plantas, al ser sésiles, requieren mecanismos que modulen la expresión génica de manera rápida y precisa permitiendo adaptar su desarrollo y fisiología a los continuos cambios ambientales a los que están sometidas. Los resultados que presento en esta tesis doctoral permiten establecer un nuevo mecanismo de regulación post-transcripcional de la expresión génica en plantas. El estudio, llevado a cabo en la especie modelo *Arabidopsis thaliana* L., reveló la existencia de un total de 11 genes *LSM*, tres de ellos duplicados, que originan proteínas capaces de formar complejos heteroheptaméricos con diferente localización subcelular y función. Uno de estos complejos, formado por las proteínas LSM1-7, es citoplásmico y se localiza en los cuerpos de procesamiento (*P-bodies*) en respuesta a condiciones de estrés tales como calor, temperaturas bajas, sequía o salinidad. La caracterización funcional de la proteína LSM1, subunidad que define este complejo, reveló su papel clave durante todo el desarrollo de Arabidopsis, así como en la tolerancia a helada, sequía y altas concentraciones de sal, regulando la expresión génica a nivel post-transcripcional. El complejo LSM1-7 interacciona con distintos tránscritos promoviendo su *decapping* y posterior degradación. Este efecto primario sobre los mensajeros diana se traduce, consecuentemente, en cambios masivos de la expresión génica que determinan el correcto desarrollo de Arabidopsis y su adaptación a diferentes situaciones ambientales adversas. Sorprendentemente, nuestros resultados han puesto de manifiesto que el complejo LSM1-7 interacciona de manera selectiva con distintos tránscritos diana en función del estadio de desarrollo y/o de la situación de estrés frente a la cual la planta se vea expuesta. El estudio de los mecanismos moleculares que determinan la especificidad mediada por el complejo LSM1-7, supone un reto apasionante para futuros trabajos.

SUMMARY

Key words: Arabidopsis, post-transcripcional regulation, decapping, gene expression, development, abiotic stress

Gene expression and its regulation play an essential function on cellular differentiation process along the development of live organisms. Moreover, gene expression reprogramming is crucial for the correct adaptation to a continuous changing environment. Plants are sessile, and accordingly, have evolved sophisticated mechanism based on gene expression adjustments to rapid and precisely adequate its development and physiology to adverse situations. The results presented in this dissertation stablish a new post-transcriptional mechanism involved in the regulation of gene expression in plants. This study, performed in the plant model specie *Arabidopsis thaliana* L., revealed the existence of 11 *LSM* genes, three of them being duplicated, which encode highly conserved proteins organized in two heteroheptameric ring-shaped complexes with different subcellular localization and function. One of these complexes, formed by the LSM1-7 proteins, is cytoplasmic and accumulates at Processing Bodies (*P-bodies*) under different abiotic stress conditions as heat, low temperatures, drought or salinity. The functional characterization of LSM1 protein, the subunit that define and characterize this complex, unveiled its key role in Arabidopsis development and, moreover, in its tolerance to freezing, drought and high salt, regulating gene expression at the post-transcriptional level. LSM1-7 interacts with selected transcripts promoting its decapping and subsequent decay. This primary effect on direct mRNA targets produces, consequently, global gene expression changes ensuring the correct development of Arabidopsis and its adaptation to different adverse situations originated by abiotic stresses. Surprisingly, our results unveiled that LSM1-7 complex selectively interacts with different transcripts depending on internal or external signals. Discovering the molecular mechanisms involved in LSM1-7 specificity, constitute an exciting issue for future studies.