Running head:

ER morphogenesis driven by plant HMGR

Corresponding author:

Narciso Campos

Departament de Genètica Molecular, Centre de Recerca en Agrigenòmica (CRAG) CSIC-IRTA-UAB-UB, Bellaterra (Cerdanyola del Vallès), 08193 Barcelona, SPAIN.

Phone : (+34) 93 5636600

ncampos@ub.edu

Research area: Cell Biology

Secondary research area: Biochemistry and Metabolism

Proliferation and morphogenesis of the ER driven by the membrane domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase in plant cells[1]

Sergi Ferrero[2,3], Ricardo Enrique Grados-Torrez[2], Pablo Leivar[4], Meritxell Antolín-Llovera[5], Carmen López-Iglesias, Nuria Cortadellas, Joan Carles Ferrer, Narciso Campos[*]

Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Barcelona 08028, Spain (S.F., P.L., M.A.Ll., J.C.F., N. Campos); Departament de Genètica Molecular, Centre de Recerca en Agrigenòmica (CRAG) CSIC-IRTA-UAB-UB, Bellaterra (Cerdanyola del Vallès), 08193 Barcelona, Spain (R.E.G.T., N. Campos); Crio-Microscopia Electrònica, Centres Científics i Tecnològics, Campus Pedralbes, Universitat de Barcelona, 08028 Barcelona, Spain (C.L.I.); and Unitat de Microscopia Electrònica, Centres Científics i Tecnològics, Campus Casanova, Universitat de Barcelona, 08036 Barcelona, Spain (N. Cortadellas).

One-sentence summary:

The membrane domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase contributes to the morphogenesis of the endoplasmic reticulum in plant cells.

[1] This work was supported by grants of the Spanish Government (BMC2003-03450, BFU2006-14655 and BFU2011-24208 to N. Campos and CSD2007-00036 to CRAG), including FEDER funds, and a grant from the Generalitat de Catalunya (2014SGR1434). S.F., R.E.G.T., P.L. and M.A.Ll., had predoctoral scholarships, respectively, from MCTE, MAEC-AECID, CIRIT and MECD.

[2] These authors contributed equally to the article.

[3] Present address: Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, 20133 Milan, Italy.

[4] Present address: Departament de Bioenginyeria IQS Universitat Ramon Llull, 08017 Barcelona, Spain.

[5] Present address: Genetics, Department of Biology, University of Munich (LMU), 82152 Munich-Martinsried, Germany.

[*] Address correspondence to ncampos@ub.edu

Material distribution footnote:

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.plantphysiol.org) is: Narciso Campos (ncampos@ub.edu).

Abstract

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) has a key regulatory role in the mevalonate pathway for isoprenoid biosynthesis and is composed of an ER-anchoring membrane domain with low sequence similarity among eukaryotic kingdoms and a conserved cytosolic catalytic domain. Organized Smooth ER (OSER) structures are common formations of hypertrophied tightly packed ER membranes, devoted to specific biosynthetic and secretory functions, the biogenesis of which remains largely unexplored. We show that the membrane domain of plant HMGR suffices to trigger ER proliferation and OSER biogenesis. The proliferating membranes become highly enriched in HMGR protein, yet they do not accumulate sterols, indicating a morphogenetic rather than a metabolic role for HMGR. The N-terminal MDVRRRPP motif present in most plant HMGR isoforms is not required for retention in the ER, as previously proposed, but functions as ER morphogenic signal. Plant OSER structures are morphologically similar to those of animal cells, emerge from tripartite ER junctions and mainly build up beside the nuclear envelope, indicating conserved OSER biogenesis in high eukaryotes. Factors other than the OSER-inducing HMGR construct mediate the tight apposition of the proliferating membranes, implying separate ER proliferation and membrane association steps. Over-expression of the membrane domain of *Arabidopsis thaliana* HMGR leads to ER hypertrophy in every tested cell type and plant species, whereas the knock out of the *HMG1* gene from *A. thaliana* causes ER aggregation at the nuclear envelope. Our results show that the membrane domain of HMGR contributes to ER morphogenesis in plant cells.

Introduction

The endoplasmic reticulum (ER) consists of a cytosolic dynamic weft of tubules and cisternae, with varying general shape and extension, continuously adapted to the changing demands of its functional commitment (Staehelin, 1997; Federovitch et al., 2005; Stefano et al., 2014). A high increase in the number and extension of ER membranes (ER hypertrophy) is a common feature of eukaryotic cells specialized in biosynthesis and secretion of metabolites and proteins. This is, for instance, the case of plant gland cells involved in the production of diverse isoprenoid products (Schnepf, 1974) and mammalian adrenocortical cells involved in steroid hormone biosynthesis (Fawcett, 1981). Such processes require elevated levels of ER-resident enzymes like HMG-CoA reductase (HMGR), cytochrome b5 and cytochrome P-450 (Boutanaev et al., 2014; Storbeck et al., 2014). Interestingly, the overexpression of these enzymes in yeast or mammalian cells induces proliferation and accumulation of ER membranes that, at the electron microscope, are visualized as stacked membranes (lamellae or karmellae), concentric membrane associations (whorled ER) or ordered arrays of membranes with hexagonal or cubic symmetry (crystalloid ER) (Jingami et al., 1987; Wright et al., 1988). These structures have been collectively named organized smooth ER (OSER) (Snapp et al., 2003). OSER structures are natural formations broadly distributed in animal and plant cells (Snapp, 2005; Almsherqi et al., 2009) and can be induced in yeast and mammalian cells by overexpression of about a dozen endogenous ER-membrane proteins (Sandig et al., 1999). Overexpression studies led to the proposal that OSER structures are caused by membrane zippering due to oligomerization of the expressed protein (Takei et al., 1994; Gong et al., 1996; Yamamoto et al., 1996; Fukuda et al., 2001; Li et al., 2003; Snapp et al., 2003; Lingwood et al., 2009; Costantini et al., 2012), but other biochemical processes are likely involved in OSER biogenesis. The huge membrane proliferation observed upon expression of OSER-inducing proteins, such as yeast or human HMGR (Jingami et al., 1987; Wright et al., 1988) or diverse cytochrome P-450 variants (Sandig et al., 1999), requires very active lipid and protein biosynthesis and cannot be explained by mere oligomerization. In spite of a long-lasting research, the mechanisms and signals triggering ER proliferation and OSER biogenesis remain elusive (Wright, 1993; Federovitch et al., 2005). Particular attention has

been paid to identification of sequence or structural features in the overexpressed protein critical for its OSER-inducing effect (Jingami et al., 1987; Vergères et al., 1993; Parrish et al., 1995; Yamamoto et al., 1996; Profant et al., 1999; Profant et al., 2000). However, the work to date has not allowed the identification of broadly conserved peptide or structural motifs that function as a signal to trigger ER proliferation and morphogenesis.

Eukaryotic HMGR has a key regulatory role in the mevalonate pathway for isoprenoid biosynthesis (Burg and Espenshade, 2011; Rodríguez-Concepción et al., 2013). Isoprenoid products derived from the pathway are involved in diverse essential functions, including membrane biogenesis (sterols), control of growth and development (steroid hormones, cytokinins), protein prenylation (farnesyl and geranyl groups), protein glycosylation (dolichols) and respiration (ubiquinones). In plants, the mevalonate pathway also provides a wide variety of compounds required for defense or positive interactions with other organisms (Chappell, 1995). HMGR has a bipartite structure formed by an N-terminal membrane domain, with low sequence similarity among eukaryotic kingdoms, and a highly conserved catalytic domain, the two connected by a flexible non-conserved linker region (Liscum et al., 1985; Basson et al., 1988; Campos and Boronat, 1995). HMGR is tetrameric in animals and plants, due to oligomerization of the catalytic domain (Bach et al., 1986; Wititsuwannakul et al., 1990; Istvan et al., 2000). The membrane domain of plant HMGR, composed of two conserved hydrophobic segments, a short lumenal loop and a cytosolic N-terminal region (Campos and Boronat, 1995), is fairly small in comparison with those of yeast and human HMGR, which are predicted to have eight trans-membrane sequences (Olender and Simoni, 1992; Profant et al., 2000). The N-terminal region is very diverged among plant HMGR isoforms, but contains a highly conserved N-terminal arginine motif that fits the consensus of ER-retention signal described in animal type II membrane proteins (Campos and Boronat, 1995). In spite of their structural differences, all eukaryotic HMGRs coincide in basic functional features. The membrane domain of yeast and human HMGR determine the targeting, turnover and capacity to induce ER proliferation, therefore controlling the catalytic activity of the appended domain (Gil et al., 1985; Jingami et al., 1987; Skalnik et al., 1988; Olender and Simoni, 1992; Parrish et al., 1995; Hampton et al., 1996). Conversely, a properly folded cytosolic domain is required to trigger karmellae biogenesis in yeast or to slow-down regulated HMGR degradation in human cells,

indicating that the tertiary structure and function of the membrane domain is influenced by the oligomerization state of the catalytic domain (Cheng et al., 1999; Profant et al., 1999). Thus in yeast and animal cells, membrane and catalytic HMGR domains show interdependent function.

In *Arabidopsis thaliana*, two genes, *HMG1* and *HMG2*, encode three HMGR isoforms: HMGR1S, HMGR1L and HMGR2 (Enjuto et al., 1994; Lumbreras et al., 1995). Isozymes HMGR1S and HMGR1L are identical in sequence, but HMGR1L has an N-terminal extension of 50 amino acid residues (1L-extra region). HMGR1S transcript is expressed in the whole plant, whereas those encoding HMGR2 or HMGR1L are detected at a much lower level and only in meristems, seedlings, roots and inflorescences (Enjuto et al., 1994; Enjuto et al., 1995; Lumbreras et al., 1995). Chimeras formed by the membrane domain of plant HMGR fused to GFP localize in the ER network, but accumulate in highly fluorescent cytosolic bodies (Leivar et al., 2005; Merret et al., 2007). In the present work we show that these bodies are OSER structures. Expression of the membrane domain of HMGR1S or HMGR2, fused to GFP, leads to reversible formation of OSER structures in every cell type of diverse plant species. The conserved N-terminal arginine motif is not an ER retention signal, but is required to induce ER proliferation and morphogenesis. In addition, the knock of the *HMG1* gene from *A. thaliana* causes severe defects in ER morphology around the nucleus. These results indicate that the membrane domain of plant HMGR is naturally involved in the morphogenesis of the ER.

Results

1S:GFP and 2:GFP induce biogenesis of ER aggregates, but 1L:GFP is retained in the ER network

Plant HMGR is primarily targeted to the ER by interaction of the highly conserved hydrophobic sequences of its membrane domain with the Signal Recognition Particle (SRP) (Campos and Boronat, 1995). To find out whether the less conserved regions flanking these sequences might influence the final subcellular location, we designed chimeras of the three *A. thaliana* HMGR isoforms, carrying the fluorescent marker GFP instead of the catalytic domain (Fig. 1, panel A). Chimeras 1S:GFP, 1L:GFP and 2:GFP contain, respectively, the membrane domain of *A. thaliana* HMGR1S, HMGR1L and HMGR2. The chimeras were transiently expressed by biolistic transfection or agroinfection in different cell types from *A. thaliana*, *Nicotiana benthamiana, Allium cepa* (onion) and *Allium porrum* (leek). In epidermal, vascular, mesophyll, trichome and culture cells from *A. thaliana* and epidermal cells from onion and leek, 1S:GFP and 2:GFP are detected in the ER network, but mainly accumulate in highly fluorescent vesicular structures, which remain close to the ER (Fig. 1, B-E; Supplemental Fig. S1, A-D; Supplemental Fig. S2, A-C; Supplemental Fig. S3, A-F). In all assayed systems, these structures emerge as small spherical bodies in three-branch junctions of the cortical ER or beside the nuclear envelope (Fig. 1, B-D; Supplemental Fig. S2, A-C; Supplemental Fig. S3, A-F) and remain physically connected to the ER network, as confirmed by 3D reconstruction of confocal microscopy images (Supplemental Movie S1). They can become large fluorescent bodies usually located near the nucleus (Fig. 1D). In transfected *N. benthamiana* leaves, 1S:GFP was expressed at high levels in epidermal cells and mostly accumulated in large densely packed structures around the nucleus (Fig. 1G). In parallel transfections of *A. thaliana*, *N. benthamiana* and onion, the ER lumen markers GFP-KDEL and GFP-HDEL outlined an ER network characteristic of the targeted cell type (Fig. 1I; Supplemental Fig. S1E; Supplemental Fig. S2E; Supplemental Fig. S3I), confirming that the aggregates were formed *de novo* upon 1S:GFP or 2:GFP expression. In contrast to 1S:GFP and 2:GFP, the 1L:GFP chimera was exclusively found in the ER network in all transfected plant systems, which included epidermal cells from *A. thaliana*, *N. benthamiana* and onion, and undifferentiated cell line from *A. thaliana* (Fig. 1, F and H;

Supplemental Fig. S2D; Supplemental Fig. S3, G and H). Since 1S:GFP and 1L:GFP behave differently, formation of the ER-derived aggregates is likely prevented by the 1L-extra region.

The physical connection of the 1S:GFP-containing aggregates to the ER network suggested but did not prove that they are part of the ER. To examine their identity, we co-transfected 1S:GFP with T3RE, which is a DsRed-derivative targeted to the ER lumen (Forés et al., 2006). In *N. benthamiana*, *A. thaliana* and onion epidermal cells, there was a perfect match between 1S:GFP and T3RE fluorescence in the ER network, nuclear envelope and spherical structures (Fig. 1, J-L; Supplemental Fig. S4). Since T3RE carries a functional ER-retention motif (C-terminal KDEL), the physical coincidence implies that the 1S:GFP-containing aggregates belong to the ER. The 1S:GFP chimera can be used as a general marker of the altered ER: it labels all ER domains, including the induced ER-membrane aggregates.

The biogenesis of ER aggregates induced by 1S:GFP is reversible

The 1S:GFP chimera was assayed also by stable transformation of *A. thaliana* plants. Six independent transgenic lines expressing high levels of 1S:GFP in the whole plant (Fig. 2, panel A) were brought to homozygosity, to prevent segregation in progeny. Alteration of the ER morphology was general in 7-day-old 1S:GFP plants. ER-derived vesicular structures were observed, for instance, in cotyledon epidermal cells, root hairs and stomata guard cells (Fig. 2, B-D). Although the transgenic lines had a slightly delayed growth with respect to wild type, they were phenotypically normal. The observed disturbance of the ER structure did not block ER function and plant development. As in the transient expression assays, 1S:GFP aggregates developed from ER network junctions (Fig. 2E) and attained the largest size beside the nuclear envelope (Fig. 2, B-D). Interestingly, 1S:GFP expression vanished between weeks 3 and 4 of plant development (Fig. 2F), but reproducibly reappeared upon seed germination, at least until generation five. The ER was altered in cells still expressing 1S:GFP (Fig. 2G), but had normal network structure in cells without the chimera, as indicated by transient expression of lumenal ER markers (Fig. 2, H and I). Biolistic transfection with the ER marker was conducted shortly after disappearance of 1S:GFP, ruling out that the observed network could belong to a newly differentiated cell.

The transfected leaves had the expected anatomy with no signs of plant tissue degeneration or regeneration (Fig. 2F). We conclude that the loss of 1S:GFP allowed progressive recovery of normal ER architecture, which was altered again in the next generation, due to reappearance of the chimera. ER disturbance driven by 1S:GFP does not irreversibly affect the organelle and is compatible with cell integrity and function.

The 1S:GFP-induced ER aggregates are OSER structures that result from membrane proliferation

To further characterize the ER-derived vesicular structures, we analyzed *N. benthamiana* and *A. thaliana* 1S:GFP-expressing organs by transmission electron microscopy (EM). In *N. benthamiana* epidermal cells, 1S-GFP expression correlates with the appearance of densely packed ER-membrane aggregates (Fig. 3, panels A and B), which were never seen in non-transfected organs. The hypertrophied ER had ordered membranes with crystalloid, lamellar or whorled patterns (Fig. 3B), as the OSER structures previously described in animal cells (Snapp et al., 2003). Also coincident with them, 1S:GFP-induced OSER structures have a narrow cytosolic space of about 10 nm between membranes, whereas the broader areas correspond to the ER lumen (Fig. 3, D and E). Ribosomes are excluded from the aggregates, which only contain smooth endoplasmic reticulum (Fig. 3, B, D and E). Similar smooth ER patterns, with narrow cytosolic space, were observed in all organs and cell types of transgenic 1S:GFP *A. thaliana* plants (Fig. 3, F-I). ER regions with crystalloid, whorled or lamellar membranes were interconnected in the same aggregate, indicating that the pattern may depend on the orientation of the section (Fig. 3, B, D and F-I). The crystalloid structures of both *A. thaliana* and *N. benthamiana* cells frequently appear beside the nuclear envelope (Fig. 3, C and F), in agreement with the observations at the confocal microscope. Ultrastructural and confocal microscope analyses indicate that expression of 1S:GFP or 2:GFP leads to a high increase of the amount of ER membranes. The 1S:GFP and 2:GFP chimeras are very effective inducers of membrane proliferation and OSER biogenesis in plant cells.

The OSER structures have high levels of 1S:GFP and HMGR but low sterol content

To confirm the identity of 1S:GFP-derived OSER structures and determine whether they also contain endogenous HMGR, we performed double immunocytochemical analyses on transgenic 1S:GFP *A. thaliana* plants, with polyclonal antibodies raised against GFP or the catalytic domain of HMGR1. For a panoramic view of single cells, we first ran whole mount immunocytochemistry on 6-day-old seedlings. In cotyledon parenchymal cells, signals corresponding to 1S:GFP and endogenous HMGR, fluorescing in green and red respectively, physically coincided in an irregular mass of ER around the blue labeled nucleus (Fig. 4, panels A-C). The ER was concentrated surrounding this organelle, with poor cortical ER (Fig. 4A).

The subcellular location of 1S:GFP and endogenous HMGR were further analyzed by immunocytochemical ultrastructural studies. In leaf epidermal cells of the transgenic *A. thaliana* seedlings, 1S:GFP and endogenous HMGR were detected at high densities on membranes of ER-derived aggregates (Fig. 4, D and E). Mixed dual labeling was observed in crystalloid, whorled and lamellar ER aggregates, in the interconnecting ER network and in the nuclear envelope (Fig. 4, E-G). No signal was detected in the control without primary antibody (Fig. 4H) nor in controls with primary and secondary antibodies in other cell compartments (Supplemental Fig. S5). The observation of normal morphologies for the Golgi, plasmalemma, mitochondria and chloroplasts indicates that membrane proliferation induced by 1S:GFP is exclusive of the ER (Supplemental Fig. S5). Our results indicate that 1S:GFP and endogenous HMGR co-localize in all regions of the ER, but accumulate mainly in the ER-derived aggregates, to which we refer as *ER-HMGR domains*. The immunodetection of GFP shows that these domains correspond to the highly fluorescent bodies identified in the confocal microscope. There is full coincidence in subcellular location between 1S:GFP and endogenous HMGR in both confocal and EM, indicating that the elimination of the catalytic domain does not alter the subcellular distribution of the HMGR protein, which is entirely determined by its membrane domain.

The high abundance of endogenous HMGR in 1S:GFP-induced OSER structures suggested that HMGR accumulates at higher levels in transgenic than in wild type plants. To examine this hypothesis, crude extracts from *A. thaliana* seedlings were submitted to immunoblot

with an antibody specific for the HMGR catalytic domain. As shown in Figure 4I, the HMGR protein level was about 3 times higher in transgenic 1S:GFP plants than in the wild type. To ascertain whether this increase was a consequence of elevated transcript levels, we ran qRT-PCR with separate primer sets specific for *HMG1* and *HMG2* (Fig. 4I). We found that the total HMGR transcript did not increase, but consistently decreased, in the transgenic 1S:GFP plants. Thus, the increase in HMGR protein was not due to higher transcript levels. The 1S:GFP chimera and endogenous HMGR accumulate in OSER structures induced by the membrane domain of HMGR1S, which has a self-stabilization effect. To find out whether the higher HMGR level caused sterol accumulation, we submitted roots of the transgenic plants to sterol-specific staining with filipin III (Boutté et al., 2011). Although the filipin signal was evident in the plasmalemma and some intracellular structures, it was nearly undetectable in 1S:GFP-induced OSER structures (Fig. 4, J-L), indicating very low levels of sterols with unreacted 3'-β-OH group. The main role of plant HMGR in OSER biogenesis does not seem to be metabolic but morphogenetic.

The membrane domain of HMGR1S suffices to trigger OSER biogenesis

It was previously shown that expression of dimerizing GFP fused to the cytoplasmic domain of otherwise passive ER resident proteins causes ER membrane stacking and OSER biogenesis and that equivalent constructs with a monomeric GFP variant do not have this morphogenic effect (Snapp et al., 2003; Costantini et al., 2012). To find out whether dimerization of GFP was contributing to the OSER inducing capacity of 1S:GFP in plant cells, we designed the 1S:GFPm chimera containing monomeric GFP. Of the three single amino acid changes known to prevent GFP dimerization (Zacharias et al., 2002), we chose the A206K substitution because it has the lowest dimerization capacity. Its Kd is about 650 times higher than that of the original GFP, making the corresponding dimer essentially undetectable (Zacharias et al., 2002). The morphogenic capacity of 1S:GFPm was first analyzed by stable transformation of the *A. thaliana* SALK_061790 knockout mutant (Myouga et al., 2010), which does not express HMGR1S nor HMGR1L. In SALK_061790 parenchymal cells, 1S:GFPm expression converted the ER network into perinuclear and cortical ER aggregates containing the monomeric chimera, as shown by whole mount

immunocytochemical analysis (Fig. 5, panel A). No endogenous HMGR could be detected under the same conditions (Fig. 5B), confirming that the observed alterations were not caused by a sharp increase in *HMG2* expression. In leaf epidermal cells from wild type *A. thaliana*, transient expression of 1S:GFPm led to the emergence of ER-derived vesicles from ER junctions (Fig. 5C), similar to those induced by transient or stable expression of 1S:GFP (Figs. 1B and 2E). The 1S:GFPm aggregates became as large as those induced by 1S:GFP (compare Figs. 5D and 1D). The 1S:GFPm chimera accumulated also in thick ER strands, that were connected to the thin ER network (Fig. 5E). In *N. benthamiana* epidermal cells, 1S:GFPm led to formation of big perinuclear ER aggregates with radially disposed thick ER strands (Fig. 5F). Thus, the appearance of thick ER strands is reiterative in cells from different plants expressing 1S:GFPm.

To confirm that the membrane domain of HMGR1S is self-sufficient to induce OSER structures, we removed GFP from the 1S:GFP chimera. This was achieved by incorporation of two consecutive stop codons at an appropriate site of the 1S:GFP coding sequence, without altering other sequences in the expression plasmid. The resulting construct encoded a 20 kD HMGR1S fragment, named 1S, containing the N-terminal region, the two membrane-spanning segments and the linker region. In *N. benthamiana* leaf epidermal cells, the non-fluorescent 1S fragment led to formation of ER aggregates, which were visualized by co-transfected T3RE, whereas, when expressed alone, the ER lumen marker localized in the typical ER network (Fig. 5, G and H). This indicates that the membrane domain of HMGR1S devoid of an appended cytosolic domain can disturb ER morphology and induce OSER structures. ER proliferation and OSER biogenesis were not as pronounced as those induced by 1S:GFP or 1S:GFPm chimeras, probably due to lower expression or stability of the 1S fragment.

To characterize OSER structures produced in the absence of dimerizing GFP, we obtained electron micrographs of agroinfected *N. benthamiana* leaves. Ultrastructural analysis of the epidermal cells uncovered that expression of 1S:GFPm led to very active proliferation of the smooth ER that was converted to interconnected crystalloid, lamellar and whorled aggregates, equivalent to those generated by 1S:GFP (Fig. 5, I-L). Particular ultrastructural features, such as exclusion of ribosomes from the aggregate and the narrow and regular

cytosolic space between membranes (Fig. 5, K and L), also coincided with those of 1S:GFP-induced OSER formations. The apposition of the cytosolic side of the ER membranes is remarkable, since monomeric GFP cannot contribute to the close interaction. Most likely, other ER-resident proteins accomplished this task. We conclude that expression of 1S:GFPm in plant cells leads to ER proliferation and association of the accumulated membranes rendering typical OSER structures.

The conserved N-terminal motif of plant HMGR is an OSER-inducing signal

It was previously suggested that the arginine motif conserved in the N-terminal region of plant HMGR might be an ER-retention signal (Campos and Boronat, 1995; Merret et al., 2007). As a first step to examine its function, we searched for all available N-terminal plant HMGR-encoding sequences in the NCBI database. To avoid overestimating variability, only full length cDNAs and ESTs confirmed by at least two independent entries were considered. We found 305 non-redundant HMGR sequences from 158 species and 117 genera of mono and dicotyledonae, which broadly represent the flowering plant kingdom (Supplemental Data Set S1). In these sequences, the three-arginine tag is only part of a longer motif consisting of the N-terminal methionine, one or two acidic residues, a hydrophobic residue, the three arginines and two prolines (Fig. 6, panel A). Two-hundred seventy three of the 305 plant HMGR isoforms (90 %) contain this canonical sequence (Supplemental Data Set S1). From them, 194 sequences keep all three arginines, whereas 79 have introduced one or two amino acid substitutions. The most repeated variations of the three-arginine tag, written in one letter code, are: HRR (17 sequences), RQR (9), SRR (8), RRK (7), RRL (4), RRQ (4), HRK (3) and RRG (3). These variants keep at least two arginines or three basic residues, indicating sequence constraints for a conserved function.

To examine the role of the conserved N-terminal motif, we obtained new variants of 1S:GFP, in which the DLRRR sequence was replaced by a single glycine residue (construct ΔDLRRR-1S:GFP) or the three arginines were replaced by three alanines (construct ΔRRR-1S:GFP) (Fig. 6). The chimeras were transiently expressed in leaf and cell culture from *A. thaliana* or *N. benthamiana*. In epidermal, vascular or *in vitro* cultured cells, both mutated variants localized in the cortical ER network (Fig. 6, B, D, F and H). No OSER

structures were induced by ΔDLRRR-1S:GFP nor by ΔRRR-1S:GFP, in spite of the long lasting expression, which, depending of the system, was prolonged for 18 to 96 h. Under the same conditions, the 1S:GFP chimera induced typical OSER structures (Fig. 6, C, E, G and I). We conclude that the conserved arginine motif is required for ER proliferation and OSER biogenesis triggered by 1S:GFP, but not for retention in the ER, which should be mediated by other elements of the N-terminal domain. In plant HMGR, the conserved N-terminal motif signals ER proliferation and morphogenesis.

In *A. thaliana*, the disruption of the *HMG1* gene causes ER aggregation around the nucleus

The requirement of a highly conserved HMGR motif for OSER biogenesis in plant cells and the OSER-inducing capacity of this protein in the three eukaryotic kingdoms, strongly suggested that HMGR has a natural role in the morphogenesis of the ER. To further examine this hypothesis, we determined ER morphology in the SALK_061790 line from *A. thaliana*, which carries a T-DNA insertion in the *HMG1* gene and is knockout for HMGR1S and HMGR1L. We transformed this line with GFP-HDEL to trace the ER lumen. Transformation was performed in heterozygous SALK_061790 plants and the morphology of the ER was studied in generation T2, which had a segregating T-DNA insertion genotype. This allowed comparison of the three *HMG1* allelic combinations on the same genetic background. The ER was characterized in cells from the root cortex of seven- to ten-day-old plants. At this age, the homozygous mutant was at the two-cotyledon stage and could be easily distinguished from homozygous wild type and heterozygous plants, which already had true leaves (Fig. 7H). We examined the ER in the cortical and nuclear ER of root cells from 36 mutant and 55 wild type plants. A total of 406 Z projection images were obtained, 201 from the mutant and 205 from the wild type, with reproducible ER morphologies. In the cortex from homozygous wild type and heterozygous plants, the GFP-HDEL labeling was prominent in *ER bodies* and weaker in the cortical ER network and the nuclear envelope (Fig. 7, A-C). The *ER bodies* are natural fusiform enlargements of the ER, very abundant in *A. thaliana* seedlings (Yamada et al., 2011). In the wild type, some *ER bodies* were around the nucleus, but only occasionally in contact with the nuclear envelope (Fig. 7A). However, in homozygous mutant cells *ER bodies* aggregated at the

nuclear envelope (Fig. 7, D-F). Under bright field, the nucleus and surrounding *ER bodies* formed a clearly visible rough mass (Fig. 7F), which could be identified also by DAPI staining (Fig. 7J). The differences between the wild type and the mutant are more evident in Z projection images of single nuclei (Fig. 7, G and I) and in videos generated from the corresponding individual sections (Supplemental Movies S2 and S3). A cross optical section of the root confirmed that the nuclear aggregates of *ER bodies* occurred in the cortex, just below the epidermal layer (Fig. 7, K and L). The disruption of *HMG1* gene caused concentration of *ER bodies* at the nuclear envelope, but did not affect the cortical ER network and the *ER bodies* connected to it, as observed in optical section series (compare Supplemental Movies S2 and S3). We conclude that the knock out of *HMG1* gene from *A. thaliana* selectively affects the organization of the ER around the nucleus, causing aggregation of *ER bodies*.

Discussion

We have shown that the membrane domain of *A. thaliana* HMGR is involved in the morphogenesis of the ER. On the one hand, the knock out of the *HMG1* gene from *A. thaliana*, encoding its major HMGR isoform, altered the ER morphology around the nucleus. On the other, the membrane domain of HMGR1S or HMGR2 fused to GFP induced massive ER proliferation and OSER biogenesis through a specific well-conserved sequence. The OSER structures emerged from ER junctions and mostly accumulated around the nucleus. Similar alterations were induced by 1S:GFP and 2:GFP in all tested cell types from diverse plant species, indicating a conserved mechanism. The non-dimerizing 1S:GFPm chimera was as effective as 1S:GFP in the induction of ER proliferation and membrane stacking, indicating that an appended dimerizing cytosolic domain is not required for the morphogenic effect. Expression of the membrane domain of HMGR1S alone also disturbed the ER network and generated OSER structures, confirming its sufficiency in the process. In agreement with the dispensability of the catalytic domain of HMGR, the induced OSER structures did not accumulate sterols. Our finding that sterols are barely detected in OSER structures is consistent with their expected low level in ER membranes (Korn, 1969) and indicates that sterol accumulation is not required for OSER biogenesis, in contrast to a previous proposal (Lingwood et al., 2009). Our results indicate morphogenetic rather than catalytic role for HMGR in OSER biogenesis, but the proliferated membranes could subsequently accommodate active enzyme machineries. It was previously proposed that the synthesis of different classes of isoprenoids could be mediated by enzymatic arrays or metabolons containing specific HMGR isozymes in particular ER domains (Chappell, 1995). Plant and animal cells engaged in high production of definite isoprenoid products typically contain OSER structures (Schnepf, 1974; Fawcett, 1981) that may contain the predicted metabolons.

We found that the three arginine motif conserved in the N-terminal region of plant HMGR is an absolute requirement to induce ER proliferation and morphogenesis. In animal cells, arginine residues located between positions 2 and 5 of type II membrane proteins constitute an ER-retention signal (Schutze et al., 1994) and a motif of arginine residues close to the N-terminus was shown to accomplish a ER-retention role also in plant cells (Boulaflous et al.,

2009). In contrast, the arginine tag of plant HMGR is not required for retention in the ER, but represents an ER-morphogenic signal. Nevertheless, the three basic residues are only a part of the N-terminal motif conserved in plant HMGR, and the two hydrophobic segments and the short lumenal region of the membrane domain are also highly conserved (Campos and Boronat, 1995). These elements may also contribute to HMGR-driven ER proliferation and morphogenesis. The requirement of the three arginine sequence for ER morphogenesis indicates that the same motif identified in 194 out of the 305 analyzed plant HMGRs (64 % of the total) should accomplish an equivalent role. We have experimentally confirmed *A. thaliana* HMGR1S and HMGR2 as members of this group. Other HMGR variants carrying two arginine residues out of the three arginine positions or with conservative changes in the basic motif (additional 26 %) will likely perform the same or very similar function. However, those HMGRs with no N-terminal arginine motif (remaining 10 %), represented by *A. thaliana* HMGR1L, may be unable to induce ER proliferation and OSER biogenesis. Thus, ER morphology may be regulated by expression of different HMGR isoforms in plant cells. The fact that OSER biogenesis is driven by plant HMGR through conserved sequences, its occurrence in all tested plants and cell types, its reversibility and its compatibility with cell function and plant development reinforce that ER morphogenesis is naturally controlled by HMGR. This was confirmed experimentally in *A. thaliana*, where the disruption of the *HMG1* gene severely affected the morphology of the nuclear ER.

In the three eukaryotic kingdoms, HMGR exerts key regulation in sterol biosynthesis through its catalytic domain and triggers ER proliferation and OSER biogenesis through its membrane domain. This coincidence has been preserved along evolution, underlining its importance for eukaryotic life. Remarkably, the membrane domain of HMGR has retained its morphogenic capacity, in spite of low sequence conservation. OSER biogenesis driven by HMGR was demonstrated here in plants and was observed previously in mammalian cells and yeast, in the three cases using homologous expression systems (Jingami et al., 1987; Wright et al., 1988). In addition, OSER structures were induced by expression of yeast HMGR in mammalian cells or mammalian HMGR in yeast (Wright et al., 1990), indicating common mechanisms in this morphogenic phenomenon. Also conserved is the subcellular location for ER proliferation. OSER biogenesis driven by HMGR has been shown to initiate at the nuclear envelope in mammalian cells (Pathak et al., 1986) and

HMGR-induced lamellae (thus named karmellae) also appear around the nucleus in yeast (Wright et al., 1988). In plant cells, OSER structures emerge from ER network junctions and around the nucleus, where they mostly accumulate. The morphogenic action of HMGR at the nuclear envelope was confirmed by disruption of *HMG1* gene from *A. thaliana*, which caused ER aggregation at this site. The fact that plant HMGR requires a highly conserved motif to control morphogenesis of the ER suggests that other eukaryotic HMGRs may have alternative sequences with equivalent function.

OSER structures induced by 1S:GFP or 1S:GFPm in plant cells are similar to those observed in animals. Higher eukaryote OSER structures are formed by tightly packed interconnected regions of crystalloid, lamellar and whorled smooth ER membranes and are morphologically more complex that the lamellar OSER formations observed in yeast. Also in a close similarity, animal and plant cell OSER structures contain membrane stacks with two distinct alternating spaces: the electron-translucent lumen, which shows a general ordered pattern, but can be distended and disordered in some regions, and the more electron-dense narrow cytosolic area, delimited by adjacent cisternae, which has a very constant width. The electron-dense aspect and even spacing of the cytosolic area indicate accumulation of regularly disposed material, likely proteins. It was suggested that the width of the cytosolic space depends of the size of the overexpressed OSER-inducing protein (Snapp et al., 2003), but this is not the case in OSER structures induced by plant HMGR. Since 1S:GFPm does not dimerize, other ER-resident or cytosolic ER-interacting proteins should be involved in the close association of membranes. Interestingly, freeze-fracture EM of transfected human cells uncovered a high density of intramembrane particles in the cytosolic face of the OSER membranes and a much lower number of similar structures in the lumenal face (Anderson et al., 1983). It is tempting to speculate that the observed protoplasmic particles may correspond to protein complexes mediating membrane apposition.

It was previously noticed that the formation of OSER structures in mammalian cells correlated with the expression level of the OSER-inducing protein. When cytochrome b5 was highly expressed, it induced OSER structures, but at lower expression, the ER network was not disturbed (Snapp et al., 2003). Thus, it was argued that OSER biogenesis merely

results from over-expression of ER-membrane proteins (Yamamoto et al., 1996; Li et al., 2003; Snapp et al., 2003). Certainly, OSER biogenesis induced by 1S:GFP is associated to a high accumulation of the chimera. However, we found that OSER structures also accumulate endogenous HMGR protein, with concomitantly reduced HMGR transcript levels. Thus, the membrane availability induced by 1S:GFP somehow stabilizes the chimera and also the endogenous HMGR, which was previously found in several plants to be submitted to ongoing degradative processes (Korth et al., 2000; Leivar et al., 2011; Pollier et al., 2013). Other endogenous and heterologous ER membrane proteins are also stabilized in OSER structures (Kochevar and Anderson, 1987; McLaughlin et al., 1999). Chimeras 1L:GFP, ΔDLRRR1S:GFP and ΔRRR1S:GFP are nearly identical to 1S:GFP, with the only difference of the naturally extended N-terminal region or the elimination of the conserved N-terminal motif. In agreement with the stabilization of HMGR in OSER structures, the observed lower expression levels of 1L:GFP, ΔDLRRR1S:GFP and ΔRRR1S:GFP might not be the cause but a consequence of their inability to induce ER proliferation and morphogenesis.

Our data indicate that ER proliferation and OSER biogenesis can be induced by ER-resident proteins, such as HMGR, carrying conserved short peptide motifs that function as ER morphogenetic signals. Previous work showed that overexpression of oligomerizing ER proteins leads to conversion of preexisting ER into OSER structures, without significant membrane proliferation (Snapp et al., 2003). On this basis, we propose a two-step model for OSER biogenesis in eukaryotic cells: 1/ ER proliferation induced by definite sequences and structural elements of particular ER-resident proteins, such as HMGR; and 2/ membrane stacking promoted by oligomerization of ER-resident proteins. This model would allow induction of OSER structures by over-expression of oligomerizing ER proteins, skipping the first of the two proposed steps. Abundant ER-resident proteins, not necessarily the one inducing ER proliferation, could mediate the stacking of membranes. In support of this, OSER biogenesis was triggered by expression of 1S:GFPm or 1S, which do not have a cytosolic oligomerizing domain. The 1S-GFPm OSER structures had tightly stacked ER membranes, with narrow cytosolic space, undistinguishable from those induced by 1S:GFP in plant cells (this work) or by other oligomerizing proteins in animal cells (Snapp et al., 2003). The stabilization of ER-resident proteins in OSER structures, detected

here for endogenous HMGR, could be favored by the availability of membrane space or the isolation from external proteases. The stabilized proteins would then contribute to OSER biogenesis by promoting membrane association. The membrane stacking step might be energy-dependent, as ATP deprivation severely disrupted the ordered pattern of proliferated ER membranes (Lingwood et al., 2009). Sustained expression of the OSER-inducing protein may be needed to maintain OSER structures, since we found that disappearance of 1S:GFP in plant cells allows reversion to normal ER network. Thus, our observations suggest that OSER formation and disassembly may be controlled in a simple way by determining the expression level of morphogenic HMGR isoforms.

MATERIALS AND METHODS

Plant Material

A. thaliana seeds were surface-sterilized washing three times with 70 % ethanol and three times with 100 % Ethanol, sowed in Petri dishes with MS medium (Murashige and Skoog, 1962) and vernalized at 4 °C for 3 days. Seedlings were grown under long day conditions (16 h light/8 h darkness) at 22-24 °C. *A. thaliana* plants were grown on a perlite:vermiculite:sphagnum (1:1:11 mixture) at the same light regime. Undifferentiated T87 cell line from *A. thaliana* was cultured as indicated (Axelos et al., 1992). The SALK_061790 mutant from *A. thaliana* (Myouga et al., 2010), carrying a T-DNA insertion in the first exon of gene *HMG1* (AT1G76490), was obtained from the European Arabidopsis Stock Centre (NASC). *N. benthamiana* was grown on perlite:vermiculite:sphagnum in green house under natural day conditions. Fresh onions and leeks were purchased in the market and used few hours later for biolistic transfection.

Constructs and plant transfection

For biolistic transfection, constructs were cloned in plasmid pGFPau (Leivar et al., 2005). For agroinfection or generation of *A. thaliana* transgenic plants, constructs were cloned in plasmid pPCV002 (Ferrando et al., 2001), kindly provided by Alejandro Ferrando (IBMCP, Valencia, Spain). In both plasmids, transcription is under control of the cauliflower mosaic virus (CaMV) 35S promoter, which confers high constitutive expression. Constructs were prepared by conventional PCR and cloning techniques, as indicated in Supplemental Table S1, using oligonucleotides listed in Supplemental Table S2. The pPCV002 derivatives were transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90RK (Koncz and Schell, 1986). Transgenic *A. thaliana* plants were generated by the floral dip method (Clough and Bent, 1998), using the transformed *A. tumefaciens* cells. Transient expression in *A. thaliana* leaves, onion scale leaves and leek scale leaves was achieved by biolistic transfection with pGFPau derivatives. Transient expression in *A. thaliana* T87 cell line (Ferrando et al., 2001) and *N. benthamiana* leaves (Van der Hoorn et al., 2000) was achieved by infection with *A. tumefaciens* (agroinfection) carrying the appropriate pPCV002 derivatives. Biolistic

transfection was performed with a *Biolistic PDS-1000/He system* (BioRad) with a 900 psi rupture disk (BioRad), as described (Lois et al., 2000). Samples were observed 18 to 48 h after biolistic transfection and 72 to 96 h after agroinfection.

Determination of transcript and protein levels

HMG1 and *HMG2* transcripts were quantified by qRT-PCR as described (Leivar et al., 2011), using the *At4g26410* transcript as a normalization reference. The primer sets and the amplicon size for the transcripts were as follows: *At4g26410*, primers 264F and 264R (81 bp); *HMG1*, primers 764F and 764R (85 bp); *HMG2*, primers HMG2F and HMG2R (92 bp). Primers are listed in Supplemental Table S2. HMGR protein levels were determined by immunoblot with anti-CD1-i antibody as described (Leivar et al., 2011).

Determination of ER morphology in the SALK_061790 mutant

To determine the morphology of the ER, the SALK_061790 line from *A. thaliana* carrying a T-DNA insertion in the *HMG1* gene was transformed with the GFP-HDEL marker of the ER lumen. Since the homozygous disruption of *A. thaliana HMG1* gene severely affects development and fertility, transformation was performed on heterozygous plants. Agrobacterium strain GV3101 pMP90 pVK-Her6 encoding GFP-HDEL (Batoko et al., 2000) was kindly provided by Dr. Ian Moore. Transformants were selected in generation T1 by resistance to hygromycin and the appearance of GFP fluorescence in the whole plant. In general, the fluorescence was more intense in the root. Generation T1 was self-crossed to obtain T2 seeds, which were germinated in MSx0.5 medium. After seven days of growth, homozygous mutants were still at the two cotyledon stage, whereas homozygous wild type and heterozygous plants had developed true leaves. The root was sectioned between days 7 and 10 for immediate observation at the confocal microscope and the rest of the plant was transferred to a fresh plate for further growth before confirmation of the genotype by PCR. The wild type allele was identified with primers H1.2F and H1.3R (547 bp amplification product) and the mutant allele with primers LB6 and H1.3R (358 bp product).

Stereo and Confocal Microscopy

A. thaliana seedlings were observed at a stereo microscope Olympus SZX16, with excitation at 460-495 nm and emission above 510 nm, to detect GFP and chlorophyll. *A. thaliana* seedlings were stained with filipin III (Boutté et al., 2011), DAPI (Kapuscinski, 1995) or propidium iodide (González-García et al., 2011) and observed at the confocal microscope as reported. Confocal laser microscopy was performed in spectral microscopes Olympus FV1000 (objectives UPLSAPO 60X O NA:1.35; and UPLSAPO 60X W NA:1.20) or Leica TCS SP5 (objectives HCX PL APO CS 40.0x1.25 OIL UV; HCX PL APO 63.0x1.20 W CORR UV; and HCX PL APO CS 63.0x1.20 WATER UV) at room temperature. Fluorophores were detected with the following excitation (ex.) and emission (em.) wavelengths: GFP (ex. 488 nm, em. 500-545 nm); T3RE (ex. 543 nm, em. 555-700 nm); Filipin-sterol complexes (ex. 364 nm, em. 400-484 nm), DAPI (ex. 405 nm, em. 425-490 nm); propidium iodide (ex. 559 nm, em. 570-670 nm); Alexa Fluor-488 (ex. 488 nm, em. 500-545 nm); Alexa Fluor-594 (ex. 559 nm, em. 570-670 nm). Images were acquired with software FV10-ASW 4.1 (Olympus) and LAS AF 2.7.3.9723 (Leica) and processed with software ImageJ 1.48v (http://imagej.nih.gov/ij).

Antibodies

HMGR was detected with immuno subtracted rabbit serum recognizing its catalytic domain (anti-CD1-i) (Leivar et al., 2011) as primary antibody, at 1:1000 for immunoblot, 1:500 for whole mount and 1:1000 for transmission EM. Anti rabbit IgG secondary antibodies for HMGR detection were: code NA934V (GE Healthcare) coupled to HRP, at 1:5000 for immunoblot; code Ab150068 (Abcam) coupled to Alexa fluor 594, at 1:1000 for whole mount; and code 111-205-144 (Jackson Immunoresearch) coupled to 12 nm gold particle, at 1:30 for transmission EM. GFP was detected with Ab-5450 (Abcam) as primary antibody, at 1:1000 for whole mount and transmission EM. Anti goat IgG secondary antibodies for GFP detection were: code Ab150133 (Abcam) coupled to Alexa Fluor 488, at 1:1000 for whole mount; and code 705-215-147 (Jackson Immunoresearch) coupled with 18 nm gold particle, at 1:15 for transmission EM.

Immunolocalization in whole mount

Whole mount *in situ* immunolocalization was done as described (Sauer et al., 2006), with modifications. After fixation in paraformaldehyde 4 % (w/v), seedlings were incubated in methanol to remove chlorophylls. Five cycles of freezing/thawing of the seedlings on glass slides were performed to permeate tissue, followed by incubation with 2 % (w/v) Driselase (Sigma, D8037) to allow penetration of antibodies through the plant cell wall. After blocking with 3 % (w/v) BSA solution, incubation with one or, simultaneously, two primary antibodies (anti-CD1-i at 1:500 and/or Ab-5450 at 1:1000) was performed directly on the slides. Samples were then washed with PBS and incubated with one or two secondary antibodies. Secondary antibody for HMGR detection (Abcam code Ab150068, Alexa fluor 594) was used at 1:1000. Secondary antibody for GFP detection (Abcam code Ab150133, Alexa Fluor 488) was used at 1:1000. After washing with PBS, samples were sealed for observation at the confocal microscope.

Transmission EM ultrastructural studies

Explants from *N. benthamiana* leaf or *A. thaliana* seedlings were removed under a stereomicroscope and transferred to glass vials filled with 1.5 % (v/v) paraformaldehyde and 1.5 % (v/v) glutaraldehyde in phosphate buffer pH 7.4, containing 2 mM $CaCl_2$. The vials were incubated at 4 °C for 48 h. After washing with the phosphate-$CaCl_2$ buffer, samples were post-fixed for 3 h at 4 °C with 1 % (w/v) osmium tetroxide and 0.8 % (w/v) potassium ferricyanide in the same buffer. Samples were subsequently dehydrated in acetone, infiltrated with Spurr resin during 4 days, embedded in the same resin and polymerized at 60 °C for 48 h. Cell integrity was confirmed at light microscope. Ultrathin sections were obtained using a Ultracut UC6 ultramicrotome (Leica) and mounted on Formvar-coated copper grids. Samples were stained with 2 % (w/v) uranyl acetate in water and lead citrate. Samples were observed in a JEM-1010 electron microscope (Jeol) equipped with a CCD camera SIS Megaview III and the AnalySIS software.

Transmission EM immunocytochemical studies

Explants from *A. thaliana* seedlings were cryo-immobilized by high pressure freezing, using an EM Pact (Leica) with yeast paste as filler. Freeze-substitution of frozen samples was performed in an Automatic Freeze substitution System EM AFS (Leica), with methanol containing 0.5 % (w/v) uranyl acetate, at -90 °C for 3 days. On the fourth day, the temperature was increased slowly, by 5 °C per hour, to -50 °C. At this temperature, samples were rinsed in acetone, and then infiltrated and flat embedded in Lowicryl HM20 for 10 days. Ultrathin sections were picked up on Formvar-coated nickel grids. Sample-containing grids were incubated on drops of PBS with 5 % (w/v) BSA, for 20 min at room temperature. After removal of washing solution, drops of PBS with the polyclonal primary antibody (anti-CD1-i or Ab-5450, at 1:1000) and 1 % (w/v) BSA were added and incubated for 2 h. Grids were washed three times for 30 min with a drop of PBS with 0.25 % (v/v) Tween 20 and incubated for 1 h in drops of PBS with the secondary antibody and 1 % (w/v) BSA. Secondary antibody for HMGR detection (Jackson Immunoresearch code 111-205-144, 12 nm gold particle) was used at 1:30. Secondary antibody for GFP detection (Jackson Immunoresearch code 705-215-147, 18 nm gold particle) was used at 1:15. Grids were washed three times with a drop of PBS for 5 min and twice with distilled water and air-dried. In control assays for non-specific binding of the gold-conjugated antibody, the primary antibody was omitted. Sections were stained with 2 % (w/v) uranyl acetate in water and lead citrate and observed in a JEM-1010 electron microscope (Jeol) with a SIS Mega View III CCD camera.

Bioinformatics

The N-terminal motif of plant HMGR was obtained with MEME software version 4.9.1 (Bailey et al., 2009) accessible at http://meme.nbcr.net/meme/, using the defect parameters.

Sequence data from this article can be found in the Arabidopsis Genome Initiative and GenBank databases under the following accession numbers: HMGR1L (At1g76490, AAR83122), HMGR1S (At1g76490, AAF16652), and HMGR2 (At2g17370, AAA67317).

Supplemental Data

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

Supplemental Figure S1. ER morphology after expression of 1S:GFP and 2:GFP in *A. thaliana* cells.

Supplemental Figure S2. ER morphology after expression of 1S:GFP, 2:GFP and 1L:GFP in *A. thaliana* cell culture.

Supplemental Figure S3. ER morphology after expression of 1S:GFP, 2:GFP and 1L:GFP in onion or leek epidermal cells.

Supplemental Figure S4. The 1S:GFP-containing aggregates belong to the ER.

Supplemental Figure S5. Controls of immunodetection and organelle morphology.

Supplemental Table S1. Preparation of DNA constructs.

Supplemental Table S2. Oligonucleotides.

Supplemental Data Set S1. N-terminal sequence of plant HMGR.

Supplemental Movie S1. Physical connection between the ER network and ER-derived aggregates.

Supplemental Movie S2. Morphology of the ER around the nucleus in the *A. thaliana hmg1* mutant.

Supplemental Movie S3. Morphology of the ER around the nucleus in wild type *A. thaliana*.

ACKNOWLEDGEMENTS

We thank NASC for seed stocks, Benjamin Glick, Patrick Gallois, Alejandro Ferrando and Ian Moore for plasmid gift, Montse Amenós for help in image processing and Albert Boronat for critical discussion. We also thank the staff of the Confocal Microscopy Service of CRAG, the Advanced Optic Microscopy Unit of CCiTUB (Faculty of Biology), Electron Microscopy Units of CCiTUB (Campus Casanova and Campus Pedralbes), *Serveis de Camps Experimentals* of UB and the Plant Growth Facility of CRAG, for support in microscopy and plant culture.

AUTHOR CONTRIBUTIONS

S.F. prepared and characterized 1S:GFP transgenic plants, identified the morphogenic properties of 1S:GFPm and performed whole mount analysis. R.E.G.T. finished transgenic plant characterization and whole mount analysis and performed confocal microscopy and EM studies in *Nicotiana* and *Arabidopsis*. R.E.G.T. and S.F. carried out most of the experiments and contributed equally to the work. P.L. and M.A.Ll. did the starting characterization of HMGR:GFP chimeras. C.L.I., N. Cortadellas and J.C.F. assisted with microscopy studies. M.A.Ll., R.E.G.T. and N. Campos characterized the HMGR mutant. N. Campos conceived and directed the project, contributed to microscopy and molecular biology studies, did the bioinformatic analysis and wrote the manuscript. All authors discussed results and commented on the manuscript.

LITERATURE CITED

Almsherqi ZA, Landh T, Kohlwein SD, Deng Y (2009) Chapter 6: cubic membranes the missing dimension of cell membrane organization. Int Rev Cell Mol Biol **274**: 275–342

Anderson RG, Orci L, Brown MS, Garcia-Segura LM, Goldstein JL (1983) Ultrastructural analysis of crystalloid endoplasmic reticulum in UT-1 cells and its disappearance in response to cholesterol. J Cell Sci **63**: 1–20

Axelos M, Curie C, Mazzolini L, Bardet C, Lescure B (1992) A protocol for transient gene-expression in Arabidopsis thaliana protoplasts isolated from cell-suspension cultures. Plant Physiol Biochem **30**: 123–128

Bach TJ, Rogers DH, Rudney H (1986) Detergent-solubilization, purification, and characterization of membrane-bound 3-hydroxy-3-methylglutaryl-coenzyme-A reductase from radish seedlings. Eur J Biochem **154**: 103–111

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res **37**: W202–8

Basson ME, Thorsness M, Finer-Moore J, Stroud RM, Rine J (1988) Structural and functional conservation between yeast and human 3-hydroxy-3-methylglutaryl coenzyme A reductases, the rate-limiting enzyme of sterol biosynthesis. Mol Cell Biol **8**: 3797–808

Batoko H, Zheng HQ, Hawes C, Moore I (2000) A rab1 GTPase is required for transport between the endoplasmic reticulum and golgi apparatus and for normal golgi movement in plants. Plant Cell **12**: 2201–18

Boulaflous A, Saint-Jore-Dupas C, Herranz-Gordo M-C, Pagny-Salehabadi S, Plasson C, Garidou F, Kiefer-Meyer M-C, Ritzenthaler C, Faye L, Gomord V (2009) Cytosolic N-terminal arginine-based signals together with a luminal signal target a type II membrane protein to the plant ER. BMC Plant Biol **9**: 144

Boutanaev AM, Moses T, Zi J, Nelson DR, Mugford ST, Peters RJ, Osbourn A (2014) Investigation of terpene diversification across multiple sequenced plant genomes. Proc Natl Acad Sci **112**: 201419547

Boutté Y, Men S, Grebe M (2011) Fluorescent in situ visualization of sterols in Arabidopsis roots. Nat Protoc **6**: 446–56

Burg JS, Espenshade PJ (2011) Regulation of HMG-CoA reductase in mammals and yeast. Prog Lipid Res **50**: 403–10

Campos N, Boronat A (1995) Targeting and topology in the membrane of plant 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Cell **7**: 2163–2174

Chappell J (1995) Biochemistry and Molecular Biology of the Isoprenoid Biosynthetic Pathway in Plants. Annu Rev Plant Physiol Plant Mol Biol **46**: 521–547

Cheng HH, Xu L, Kumagai H, Simoni RD (1999) Oligomerization state influences the degradation rate of 3-hydroxy-3-methylglutaryl-CoA reductase. J Biol Chem **274**: 17171–8

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16**: 735–43

Costantini LM, Fossati M, Francolini M, Snapp EL (2012) Assessing the tendency of fluorescent proteins to oligomerize under physiologic conditions. Traffic **13**: 643–9

Enjuto M, Balcells L, Campos N, Caelles C, Arró M, Boronat A (1994) Arabidopsis thaliana contains two differentially expressed 3-hydroxy-3-methylglutaryl-CoA reductase genes, which encode microsomal forms of the enzyme. Proc Natl Acad Sci U S A **91**: 927–931

Enjuto M, Lumbreras V, Marín C, Boronat A (1995) Expression of the Arabidopsis HMG2 gene, encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, is restricted to meristematic and floral tissues. Plant Cell **7**: 517–527

Fawcett DW (1981) Endoplasmatic reticulum. Cell, 2nd ed. Saunders, Philadelphia [etc.] :, pp 303–368

Federovitch CM, Ron D, Hampton RY (2005) The dynamic ER: experimental approaches and current questions. Curr Opin Cell Biol **17**: 409–14

Ferrando A, Koncz-Kálmán Z, Farràs R, Tiburcio A, Schell J, Koncz C (2001) Detection of in vivo protein interactions between Snf1-related kinase subunits with intron-tagged epitope-labelling in plants cells. Nucleic Acids Res **29**: 3685–93

Forés O, Arró M, Pahissa A, Ferrero S, Germann M, Stukey J, McDonough V, Nickels Jr. JT, Campos N, Ferrer A (2006) Arabidopsis thaliana expresses two functional isoforms of Arvp, a protein involved in the regulation of cellular lipid horneostasis. Biochim Biophys Acta-Molecular Cell Biol Lipids **1761**: 725–735

Fukuda M, Yamamoto A, Mikoshiba K (2001) Formation of crystalloid endoplasmic reticulum induced by expression of synaptotagmin lacking the conserved WHXL motif in the C terminus. Structural importance of the WHXL motif in the C2B domain. J Biol Chem **276**: 41112–9

Gil G, Faust JR, Chin DJ, Goldstein JL, Brown MS (1985) Membrane-bound domain of HMG CoA reductase is required for sterol-enhanced degradation of the enzyme. Cell **41**: 249–58

Gong FC, Giddings TH, Meehl JB, Staehelin LA, Galbraith DW (1996) Z-membranes: artificial organelles for overexpressing recombinant integral membrane proteins. Proc Natl Acad Sci U S A **93**: 2219–23

González-García M-P, Vilarrasa-Blasi J, Zhiponova M, Divol F, Mora-García S, Russinova E, Caño-Delgado AI (2011) Brassinosteroids control meristem size by promoting cell cycle progression in Arabidopsis roots. Development **138**: 849–59

Hampton RY, Koning A, Wright R, Rine J (1996) In vivo examination of membrane protein localization and degradation with green fluorescent protein. Proc Natl Acad Sci U S A **93**: 828–33

Van der Hoorn RA, Laurent F, Roth R, De Wit PJ (2000) Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis. Mol Plant Microbe Interact **13**: 439–46

Istvan ES, Palnitkar M, Buchanan SK, Deisenhofer J (2000) Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. EMBO J **19**: 819–830

Jingami H, Brown MS, Goldstein JL, Anderson RG, Luskey KL (1987) Partial deletion of membrane-bound domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase eliminates sterol-enhanced degradation and prevents formation of crystalloid endoplasmic reticulum. J Cell Biol **104**: 1693–704

Kapuscinski J (1995) DAPI: a DNA-specific fluorescent probe. Biotech Histochem **70**: 220–33

Kochevar DT, Anderson RG (1987) Purified crystalloid endoplasmic reticulum from UT-1 cells contains multiple proteins in addition to 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Biol Chem **262**: 10321–6

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. MGG Mol Gen Genet **204**: 383–396

Korn ED (1969) Cell membranes: structure and synthesis. Annu Rev Biochem **38**: 263–88

Korth KL, Jaggard DA, Dixon RA (2000) Developmental and light-regulated post-translational control of 3-hydroxy-3-methylglutaryl-CoA reductase levels in potato. Plant J **23**: 507–16

Leivar P, Antolín-Llovera M, Ferrero S, Closa M, Arró M, Ferrer A, Boronat A, Campos N (2011) Multilevel Control of Arabidopsis 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase by Protein Phosphatase 2A. Plant Cell **23**: 1494–1511

Leivar P, González VM, Castel S, Trelease RN, López-Iglesias C, Arró M, Boronat A, Campos N, Ferrer A, Fernàndez-Busquets X (2005) Subcellular localization of Arabidopsis 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Plant Physiol **137**: 57–69

Li Y, Dinsdale D, Glynn P (2003) Protein domains, catalytic activity, and subcellular distribution of neuropathy target esterase in Mammalian cells. J Biol Chem **278**: 8820–5

Lingwood D, Schuck S, Ferguson C, Gerl MJ, Simons K (2009) Generation of cubic membranes by controlled homotypic interaction of membrane proteins in the endoplasmic reticulum. J Biol Chem **284**: 12041–8

Liscum L, Finer-Moore J, Stroud RM, Luskey KL, Brown MS, Goldstein JL (1985) Domain structure of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a glycoprotein of the endoplasmic reticulum. J Biol Chem **260**: 522–30

Lois LM, Rodríguez-Concepción M, Gallego F, Campos N, Boronat A (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J **22**: 503–13

Lumbreras V, Campos N, Boronat A (1995) The use of an alternative promoter in the Arabidopsis thaliana HMG1 gene generates an messenger RNA that encodes a novel 3-hydroxy-3-methylglutaryl coenzyme A reductase isoform with an extended N-terminal region. Plant J **8**: 541–549

McLaughlin L, Burchell B, Pritchard M, Wolf CR, Friedberg T (1999) Treatment of mammalian cells with the endoplasmic reticulum-proliferator compactin strongly induces recombinant and endogenous xenobiotic metabolizing enzymes and 3-hydroxy-3-methylglutaryl-CoA reductase in vitro. J Cell Sci **112 (Pt 4**: 515–23

Merret R, Cirioni JR, Bach TJ, Hemmerlin A (2007) A serine involved in actin-dependent subcellular localization of a stress-induced tobacco BY-2 hydroxymethylglutaryl-CoA reductase isoform. FEBS Lett **581**: 5295–5299

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant **15**: 473–497

Myouga F, Akiyama K, Motohashi R, Kuromori T, Ito T, Iizumi H, Ryusui R, Sakurai T, Shinozaki K (2010) The Chloroplast Function Database: a large-scale collection of Arabidopsis Ds/Spm- or T-DNA-tagged homozygous lines for nuclear-encoded chloroplast proteins, and their systematic phenotype analysis. Plant J **61**: 529–42

Olender EH, Simoni RD (1992) The intracellular targeting and membrane topology of 3-hydroxy-3-methylglutaryl-CoA reductase. J Biol Chem **267**: 4223–35

Parrish ML, Sengstag C, Rine JD, Wright RL (1995) Identification of the sequences in HMG-CoA reductase required for karmellae assembly. Mol Biol Cell **6**: 1535–47

Pathak RK, Luskey KL, Anderson RG (1986) Biogenesis of the crystalloid endoplasmic reticulum in UT-1 cells: evidence that newly formed endoplasmic reticulum emerges from the nuclear envelope. J Cell Biol **102**: 2158–68

Pollier J, Moses T, González-Guzmán M, De Geyter N, Lippens S, Vanden Bossche R, Marhavý P, Kremer A, Morreel K, Guérin CJ, et al (2013) The protein quality control system manages plant defence compound synthesis. Nature **504**: 148–52

Profant DA, Roberts CJ, Koning AJ, Wright RL (1999) The role of the 3-hydroxy 3-methylglutaryl coenzyme A reductase cytosolic domain in karmellae biogenesis. Mol Biol Cell **10**: 3409–23

Profant DA, Roberts CJ, Wright RL (2000) Mutational analysis of the karmellae-inducing signal in Hmg1p, a yeast HMG-CoA reductase isozyme. Yeast **16**: 811–27

Rodríguez-Concepción M, Campos N, Ferrer A, Boronat A (2013) Biosynthesis of isoprenoid precursors in Arabidopsis. *In* TJ Bach, M Rohmer, eds, Isoprenoid Synth. plants Microorg. new concepts Exp. approaches. Springer Science+Business Media, New York, pp 439–456

Sandig G, Kärgel E, Menzel R, Vogel F, Zimmer T, Schunck WH (1999) Regulation of endoplasmic reticulum biogenesis in response to cytochrome P450 overproduction. Drug Metab Rev **31**: 393–410

Sauer M, Paciorek T, Benková E, Friml J (2006) Immunocytochemical techniques for whole-mount in situ protein localization in plants. Nat Protoc **1**: 98–103

Schnepf E (1974) Gland cells. *In* AW Robards, ed, Dyn. Asp. plant Ultrastruct. McGraw-Hill, London [etc.] :, pp 331–57

Schutze MP, Peterson PA, Jackson MR (1994) An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. EMBO J **13**: 1696–705

Skalnik DG, Narita H, Kent C, Simoni RD (1988) The membrane domain of 3-hydroxy-3-methylglutaryl-coenzyme A reductase confers endoplasmic reticulum localization and sterol-regulated degradation onto β-galactosidase. J Biol Chem **263**: 6836–41

Snapp E (2005) Endoplasmic reticulum biogenesis: proliferation and differentiation. *In* C Mullis, ed, Biog. Cell. Organelles. Kluwer Academic / Plenum Publishers / Landes Bioscience, Georgetown/New York, pp 63–95

Snapp EL, Hegde RS, Francolini M, Lombardo F, Colombo S, Pedrazzini E, Borgese N, Lippincott-Schwartz J (2003) Formation of stacked ER cisternae by low affinity protein interactions. J Cell Biol **163**: 257–69

Staehelin LA (1997) The plant ER: a dynamic organelle composed of a large number of discrete functional domains. Plant J **11**: 1151–65

Stefano G, Hawes C, Brandizzi F (2014) ER - the key to the highway. Curr Opin Plant Biol **22**: 30–8

Storbeck K-H, Swart AC, Fox CL, Swart P (2014) Cytochrome b5 modulates multiple reactions in steroidogenesis by diverse mechanisms. J Steroid Biochem Mol Biol. doi: 10.1016/j.jsbmb.2014.11.024

Takei K, Mignery GA, Mugnaini E, Südhof TC, De Camilli P (1994) Inositol 1,4,5-trisphosphate receptor causes formation of ER cisternal stacks in transfected fibroblasts and in cerebellar Purkinje cells. Neuron **12**: 327–42

Vergères G, Yen TS, Aggeler J, Lausier J, Waskell L (1993) A model system for studying membrane biogenesis. Overexpression of cytochrome b5 in yeast results in marked proliferation of the intracellular membrane. J Cell Sci **106**: 249–59

Wititsuwannakul R, Wititsuwannakul D, Suwanmanee P (1990) 3-Hydroxy-3-methylglutaryl coenzyme A reductase from the latex of Hevea brasiliensis. Phytochemistry **29**: 1401–1403

Wright R (1993) Insights from inducible membranes. Curr Biol **3**: 870–3

Wright R, Basson M, D'Ari L, Rine J (1988) Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. J Cell Biol **107**: 101–14

Wright R, Keller G, Gould SJ, Subramani S, Rine J (1990) Cell-type control of membrane biogenesis induced by HMG-CoA reductase. New Biol **2**: 915–21

Yamada K, Hara-Nishimura I, Nishimura M (2011) Unique defense strategy by the endoplasmic reticulum body in plants. Plant Cell Physiol **52**: 2039–2049

Yamamoto A, Masaki R, Tashiro Y (1996) Formation of crystalloid endoplasmic reticulum in COS cells upon overexpression of microsomal aldehyde dehydrogenase by cDNA transfection. J Cell Sci **109**: 1727–38

Zacharias DA, Violin JD, Newton AC, Tsien RY (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science **296**: 913–6

FIGURE LEGENDS

Figure 1. ER morphology after expression of 1S:GFP, 2:GFP and 1L:GFP in *A. thaliana* and *N. benthamiana* cells.

A, Schematic representation in the ER membrane of HMGR:GFP chimeras used in this study. Chimeras 1S:GFP, 1L:GFP and 2:GFP have the membrane domain of *A. thaliana* HMGR1S, HMGR1L and HMGR2 respectively, fused to C-terminal GFP. Construct 1S consist in the membrane domain of HMGR1S alone.

B-L, *A. thaliana* (B-F) and *N. benthamiana* (G-L) leaves were transfected with constructs encoding the HMGR chimera (1S:GFP, 2:GFP or 1L:GFP) or a control ER lumen marker (GFP-HDEL or T3RE) and visualized by confocal laser microscopy.

J-L, cotransfection with 1S:GFP and T3RE, visualized by confocal laser microscopy, as indicated.

The images are Z projections of 10 (B-F), 20 (J-L) or 50 (G-I) sections from the transfected *A. thaliana* epidermal (B, C, F), *A. thaliana* vascular (D, E) or *N. benthamiana* epidermal (G-L) cells. Bars = 10 μm.

Figure 2. Reversible formation of ER-aggregates in transgenic 1S:GFP *A. thaliana* plants.

Transgenic plants were analyzed by stereomicroscopy (A, F) and confocal laser microscopy (B-E, G-I), to detect the fluorescence of 1S:GFP in green (A-G), endogenous chlorophyll in red (F) or ER lumen markers in red or green (H, I).

A, Ten day-old transgenic seedlings have widespread 1S:GFP expression.

B-D, The chimera induces ER-derived aggregates in all cell types including leaf epidermal cells (B, E), root hairs (C) and stomata guard cells (D).

F, After three weeks of growth, 1S:GFP vanishes, allowing detection of red fluorescence from chlorophyll.

G-I, ER-derived aggregates are present in leaf sectors still expressing 1S:GFP (G) but not in areas without the chimera, as shown by biolistic transfection of ER-lumen markers (H, I).

Confocal microscope images are Z projections of 12 (B, D, E, H, I) and 21 (G) sections from the transfected cells, except that of (C), which corresponds to a single section. Bars = 3 mm (A, F); 10 μm (B-E, G-I).

Figure 3. Ultrastructural analysis of 1S:GFP-induced ER aggregates.

Agroinfected *N. benthamiana* leaves (A-E) and transgenic *A. thaliana* seedlings (F-I) expressing 1S:GFP were imaged by transmission EM.

A-E, In *N. benthamiana* leaf epidermal cells, the aggregates are composed of interconnected crystalloid, lamellar and whorled smooth ER (A, B), frequently located beside the nucleus (C). Whorled structures contain alternating lumenal (aligned dots) and more electron-dense cytosolic spaces that exclude ribosomes (white arrow heads) (D). The narrow (10 nm) electron-dense space between membranes (arrowhead) shows continuity with the cytosol (E).

F-I, In all cell types of transgenic 1S:GFP *A. thaliana* seedlings, the ER membrane aggregates also accumulate around the nucleus and have diverse interconnected patterns.

Cr: crystalloid; W: whorled; La: lamellar; N: nucleus; n: nucleolus. Bars = 2 μm (A); 0.5 μm (B); 1 μm (C); 200 nm (D); 100 nm (E); 1 μm (F); 0.5 μm (G, H, I).

Figure 4. 1S:GFP-induced OSER structures have high levels of 1S:GFP and HMGR, but low sterol content.

A-C, Whole mount immunocytochemical analysis of transgenic 1S:GFP *A. thaliana* seedlings. Immunodetection of 1S:GFP (green) and HMGR (red) in cotyledon parenchymal cells, visualized by confocal microscopy. The two proteins colocalize in ER aggregates around the DAPI-stained (blue) nucleus.

D-H, Immunocytochemical EM analysis of transgenic 1S:GFP *A. thaliana* seedlings. 1S:GFP (18 nm particle) and endogenous HMGR (12 nm particle) were immunodetected in leaf epidermal cells. The two proteins colocalize on membranes of crystalloid aggregates (D, E, G), whorled aggregates (F) and nuclear envelope (arrow head) (G). No signal is observed in the control without primary antibodies (H).

I, Quantification of *HMG1* and *HMG2* transcripts and HMGR protein in transgenic 1S:GFP seedlings. Transcript and protein levels were measured by qRT-PCR or immunoblot and are represented relative to *HMG1* transcript or HMGR1S protein of wt seedlings, respectively, as the average ± SEM of three independent experiments. Electrophoretic migration of HMGR1L (69 kD) and HMGR1S (63 kD) is indicated. Coomassie-stained Rubisco was used as a loading control and is shown bellow the immunoblot.

J-K, Sterol and 1S:GFP distribution in root cortical cells, in transgenic 1S:GFP seedlings. Samples were stained with filipin III and visualized by confocal microscopy. Free sterols show poor colocalization with 1S:GFP.

Confocal microscope images are Z projections of 10 (A-C) or 15 (J-L) sections. Bars = 10 µm (A-C); 1 µm (D); 200 nm (E, F); 0.5 µm (G, H); 30 µm (J-L).

Figure 5. OSER structures induced by 1S:GFPm and 1S fragment.

A-B, Whole mount immunocytochemical analysis of the SALK_061790 *A. thaliana* mutant stably transformed with 1S:GFPm. Immunodetection of 1S:GFPm (A) and HMGR (B) in cotyledon parenchymal cells of 1S:GFPm/SALK_061790 seedlings, visualized by confocal microscopy with DAPI-stained nuclei.

C-F, Transient expression of 1S:GFPm in epidermal cells from wild type *A. thaliana* (C-E) and *N. benthamiana* (F) leaves, visualized by confocal microscopy.

G-H, Transient co-expression of 1S fragment and T3RE ER marker (G) in epidermal cells from *N. benthamiana* leaves, using expression of T3RE alone as a control (H).

I-L, Transmission EM of epidermal cells from *N. benthamiana* leaf after transient expression of 1S:GFPm.

Confocal microscope images are Z projections of 10 (A-E), 33 (F), 15 (G) and 19 (H) sections. Bars = 10 µm (A-H); 1 µm (I); 0.5 µm (J, K); 200 nm (L).

Figure 6. Role of the N-terminal motif of plant HMGR in the morphogenesis of the ER.

A, N-terminal motif of plant HMGR deduced from non redundant cDNA and EST entries (see Supplemental Data Set S1) with MEME software (Bailey et al., 2009). Letter size is proportional to amino acid residue frequency in the referred position.

B-I, The original 1S:GFP (N-terminal sequence MDLRRRPPKPP) and its mutated variants ΔDLRRR-1S:GFP (sequence MGPPKPP) and ΔRRR-1S:GFP (sequence MDLAAAPPKPP) were transiently expressed by biolistic in *A. thaliana* leaf (B-E) or agroinfection in *A. thaliana* cell culture (F, G) and *N. benthamiana* leaf (H, I). Vascular (B, C), epidermal (D, E, H, I) and *in vitro* culture cells (F, G) were visualized by confocal microscopy.

Images are Z projection of 10 (B-G) or 50 (H, I) sections. Bars = 10 µm.

Figure 7. Effect of the disruption of the *HMG1* gene from *A. thaliana* on the morphology of the ER.

A. thaliana plants of the SALK_061790 line, heterozygous for the disrupted *HMG1* gene, were transformed with GFP-HDEL. At the segregating generation T2, the differentiation zone of roots from 7 to 10-day old plants was examined by confocal laser microscopy. Images were captured after fluorescent excitation of GFP or propidium iodide (A, D, G, I-L), under bright field (C, F) or with the merge of both channels (B, E).

A-C, Morphology of the ER in the root of heterozygous plant. The GFP-HDEL label is detected in the cortical ER, in the nuclear envelope and, more intensively, in *ER bodies*. The nuclei (indicated by arrows) appear as fusiform structures.

D-F, Morphology of the ER in the root of homozygous SALK_061790 mutant plant. The GFP-HDEL label mainly distributes in *ER bodies* around the nucleus. The nuclei (seen from their front, indicated by arrows) have a circular appearance, with an irregular outline.

G, Nuclear region in cortex cells from the SALK_061790 *hmg1* mutant. The image corresponds to a Z projection of 15 sections from the GFP channel, taken every 0.85 µm. The *ER bodies* accumulate around the nucleus, which is indicated with a yellow N. The individual sections are shown, on bright field, in Supplemental Movie S2.

H, Phenotype of SALK_061790 homozigous *hmg1* mutant (left) and wild type (right) plants 10 days after growth at 22 °C. The mutant plant has a delayed development in both aerial and root organs and is still at the two cotyledon stage. Bar = 3 mm.

I, Nuclear region in cortex cells from the wild type. The center of the nucleus is indicated with a yellow N. The image corresponds to a Z projection of 15 sections from the GFP channel, taken every 0.52 µm. The individual sections are shown on bright field in Supplemental Movie S3.

J, DAPI staining and GFP detection of homozygous mutant root. The image corresponds to a Z projection of 7 sections.

K, L, Propidium iodide staining and GFP detection of homozygous mutant root. Panel L correspond to the XZ-scanning section indicated by the dashed line of panel K. The nuclear *ER body* aggregates localize in cortex cells, just bellow the epidermal layer.

Images A-F, K and L correspond to single scanning sections with the bar indicating 20 µm. Images G, I and J are Z projections with the bar indicating 10 µm.