

Interaction of the plant glycine-rich RNA-binding protein MA16 with a novel nucleolar DEAD box RNA helicase protein from *Zea mays*

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Summary

The maize RNA-binding MA16 protein is a developmentally and environmentally regulated nucleolar protein that interacts with RNAs through complex association with several proteins. By using yeast two-hybrid screening, we identified a DEAD box RNA helicase protein from *Zea mays* that interacted with MA16, which we named *Z. mays* DEAD box RNA helicase 1 (ZmDRH1). The sequence of ZmDRH1 includes the eight RNA helicase motifs and two glycine-rich regions with arginine–glycine-rich (RGG) boxes at the amino (N)- and carboxy (C)-termini of the protein. Both MA16 and ZmDRH1 were located in the nucleus and nucleolus, and analysis of the sequence determinants for their cellular localization revealed that the region containing the RGG motifs in both proteins was necessary for nuclear/nucleolar localization. The two domains of MA16, the RNA recognition motif (RRM) and the RGG, were tested for molecular interaction with ZmDRH1. MA16 specifically interacted with ZmDRH1 through the RRM domain. A number of plant proteins and vertebrate p68/p72 RNA helicases showed evolutionary proximity to ZmDRH1. In addition, like p68, ZmDRH1 was able to interact with fibrillarin. Our data suggest that MA16, fibrillarin, and ZmDRH1 may be part of a ribonucleoprotein complex involved in ribosomal RNA (rRNA) metabolism.

Keywords: DEAD-box RNA helicase, glycine-rich RNA-binding proteins, nucleolar localization, RRM domain, RGG domain, *Zea mays*.

Introduction

Plant glycine-rich RNA-binding proteins (GRPs) are a small family of proteins that consist of two domains: one RNA recognition motif (RRM) at the protein amino (N)-terminal part followed by a carboxy (C)-terminal region composed of up to 70% glycine residues interrupted mostly by arginine or aromatic amino acid residues and containing arginine–glycine-rich (RGG) motifs (Burd and Dreyfuss, 1994). RGG domains were first identified in several nucleolar proteins such as nucleolin, NSR1, GAR1, and fibrillarin, as well as in the non-nucleolar heterogeneous nuclear ribonucleoprotein particle (hnRNP)A1/A2 (Nagai, 1996). It has been reported that RGG domains serve functions as diverse as RNA binding, protein–protein interaction, and nucleolar targeting. In fact, there is considerable amount of data, indicating that these domains may be involved in non-sequence-specific RNA interactions (Heine *et al.*, 1993;

Kiledjian and Dreyfuss, 1992). The RRM domain is comprised of 80–90 amino acids and contains two short, highly conserved segments (RNP-1 and RNP-2), within which the conserved aromatic amino acids have been shown to interact with RNA directly (Nagai *et al.*, 1995; Siomi and Dreyfuss, 1997). Although many proteins carrying the RRM have been characterized in vertebrates and yeast, much less is known about plant RNA-binding proteins, and in only few cases has their function been studied (Alba and Pages, 1998).

We have previously described the first member of the GRP family MA16, which accumulates in embryos and is involved in a variety of stress responses in the plant (Gomez *et al.*, 1988; Mortenson and Dreyfuss, 1989). Since then, homologous proteins have been found in not only other plant species but also cyanobacteria (Sato, 1995) and

vertebrates such as mice (CIRP; Nishiyama *et al.*, 1997) and humans (RBM3; Derry *et al.*, 1995). Detailed phylogenetic analysis has shown however that the cyanobacteria glycine-rich RNA-binding proteins (Rbps) and the eukaryotic GRPs do not belong to a single lineage, but that the glycine-rich domains are likely to have been added independently (Maruyama *et al.*, 1999). Interestingly, a large number of studies have reported an increase in the abundance of these GRPs under stress conditions, such as drought (Bergeron *et al.*, 1993; Carpenter *et al.*, 1994; Gomez *et al.*, 1988), salinity (Aneeta *et al.*, 2002), wounding (Sturm *et al.*, 1992), abscisic acid (ABA; Aneeta *et al.*, 2002; Bergeron *et al.*, 1993; Carpenter *et al.*, 1994; Gomez *et al.*, 1988), cold (Bergeron *et al.*, 1993; Carpenter *et al.*, 1994; Horvath and Olson, 1998), and circadian rhythm (Carpenter *et al.*, 1994; Heintzen *et al.*, 1994; Heintzen *et al.*, 1997). Thus, this pattern of expression suggests that glycine-rich proteins represent a class of RNA-binding proteins involved in general molecular responses to environmental stress mediated by post-transcriptionally regulatory mechanisms. Post-transcriptional processes play crucial roles in the overall control and regulation of eukaryotic gene expression (Siomi and Dreyfuss, 1997). Specific RNA-protein interactions have been shown to control RNA transcription, RNA processing, transport, and translation (Gesteland and Atkins, 1993; Nagai, 1992). A large number of RNA-binding proteins involved in these processes have been identified. These proteins include poly(A)-binding proteins (PABPs), required for mRNA stability, small nuclear ribonucleoprotein particle (snRNP) proteins and arginine/serine proteins (SR) involved in RNA splicing and hnRNP proteins necessary for RNA transport (Dreyfuss *et al.*, 1993; Swanson and Lamond, 1995).

Although the function of GRPs remains unknown, a differential affinity for various types of RNA has been reported. In ribohomopolymer-binding assays, proteins from maize, tobacco, and barley show a high affinity for poly(G) and poly(U) (Dunn *et al.*, 1996; Hirose *et al.*, 1993; Ludevid *et al.*, 1992), suggesting that cellular RNA ligands are likely to be enriched with G and U residues. They also bind single-stranded DNA (Dunn *et al.*, 1996; Hirose *et al.*, 1993) and are phosphorylated both *in vivo* and *in vitro* (Dunn *et al.*, 1996; Freire and Pages, 1995). We showed that MA16 has higher binding affinities to particular RNAs, including ribosomal RNA (rRNA) and its own mRNA, suggesting a possible feedback control, and immunoprecipitation experiments showed that MA16 interacts with RNAs through a complex association with several proteins (Freire and Pages, 1995).

Immunolocalization experiments have shown that GRPs are present in nuclei (Danno *et al.*, 2000; Heintzen *et al.*, 1997; Hirose *et al.*, 1994) and, as in the case of MA16 and tobacco GRP1b, in nucleolus (Alba *et al.*, 1994; Hirose *et al.*, 1994). These results suggest that GRPs may participate

in pre-rRNA-processing events. Interestingly, AtGRP-2, NsGRP-2, and RZ-1 contain, in addition to the glycine-rich and RNA-binding motifs, one or two CCHC zinc-fingers, similar to some splicing factors (Nagai, 1996). So, it is possible that these GRPs represent components of the plant-cell-splicing machinery. In addition, *in situ* hybridization experiments have revealed a higher concentration of MA16 mRNA in different expanding tissues of maize seedlings (Alba and Pages, 1998). Taken together, these results appear to indicate that glycine-rich proteins could be involved in rRNA metabolism and growth, and that they could regulate or affect these processes during environmental stresses.

In spite of the availability of data concerning the tissue expression and RNA-binding properties of GRPs, their association with other proteins or subcellular structures remains an unexplored field. In order to gain an insight into the biological function of GRPs in plants and more details about the function of MA16, we have used here the yeast two-hybrid system to recover MA16-interacting proteins. Using this approach, we have identified a novel nucleolar protein that shows homology to the DEAD box RNA helicase p68 subfamily (Lamm *et al.*, 1996), which we have named *Z. mays* DEAD box RNA helicase 1 (ZmDRH1). Both MA16 and ZmDRH1 contain RGG boxes and localize in the nucleus and nucleolus. Moreover, we have found that the nucleolar protein fibrillarin is a potential partner of ZmDRH1 *in vitro*. The data support a role of MA16 and ZmDRH1, acting as part of a molecular complex, in rRNA metabolism in the nucleolus.

Results

Subcellular targeting of MA16 protein

We have previously reported that MA16 is a nuclear/nucleolar protein in maize tissues (Alba *et al.*, 1994). To determine the sequence requirements for nuclear import and nucleolar accumulation of MA16, transient expression assays in onion epidermal cells were performed. To this aim, the entire MA16 protein, the RRM domain, or the RGG domain was fused in frame to the 5' end of the green fluorescent protein (GFP) sequence and placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. As seen in Figure 1, MA16-GFP was localized in nuclei and accumulated in nucleoli, in contrast to the control GFP, which was distributed throughout the cytoplasm and the nucleus. When we transformed onion cells using the RRM-GFP construct, the fusion protein was confined exclusively to the nuclei and was excluded from the nucleoli (Figure 1). The same pattern was obtained when the RRM domain was fused to both β -glucuronidase (GUS) and GFP (data not shown), suggesting that RRM is sufficient for nuclear

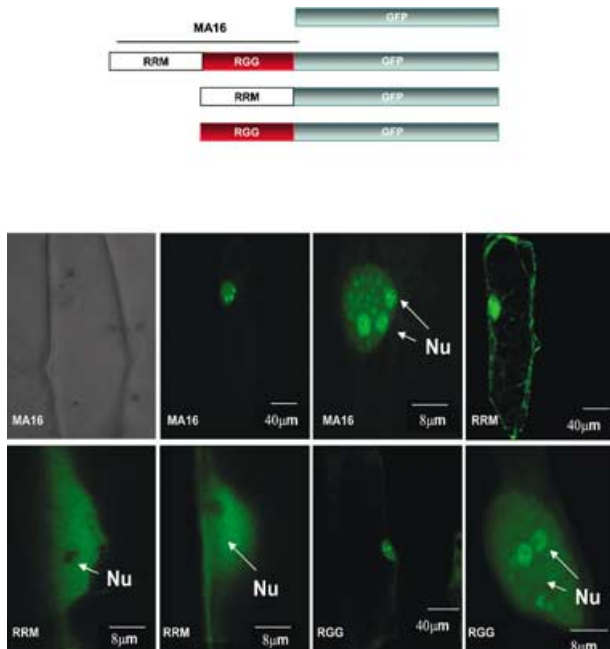


Figure 1. Subcellular localization of MA16.

(a) Schematic representation MA16 constructs used in bombardment experiments. RGG, arginine-glycine-rich domain; and RRM, RNA recognition motif.

(b) Representative images of nuclei from transiently transformed onion epidermal cells expressing GFP fusions to MA16 and RRG and RRM domains individually. The transformation was repeated at least three times for each construct. Nu, nucleoli.

targeting. Interestingly, when the RGG-GFP construct was transformed, the fusion protein was observed in both nuclei and nucleoli, suggesting that RGG domain is necessary and sufficient for both nuclear and nucleolar localization. Similar results have been obtained for the *Arabidopsis* fibrillarin (Pih *et al.*, 2000), in which the RRG domain is required for nucleolar targeting, and deletions of this domain result in a loss of nucleolus accumulation.

Presence of MA16 in nuclear ribonucleoprotein fractions

We previously reported that MA16 showed a wide spectrum of RNA-binding activities and higher affinities to particular RNAs, including rRNA and translatable mRNA sequences. Immunoprecipitation experiments suggested that MA16 interacts with RNAs through complex association with several proteins. (Alba *et al.*, 1994; Freire and Pages, 1995). These data suggest that MA16 may be forming part of an RNP. As a first step to identify MA16 interacting partners, we localized the protein in nuclear fractions of maize seedlings.

Purified *Zea mays* cell nuclei were subjected to sequential extraction of proteins. Five substantially different nuclear fractions were obtained according to their solubility in

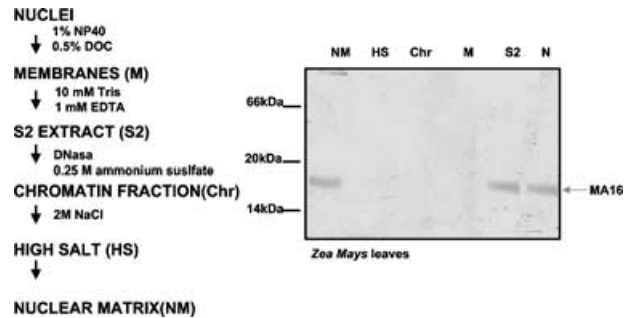


Figure 2. Detection of MA16 in nuclear fractions by Western blot.

Flow diagram of the procedure of sequential nuclear protein fractionation (de Carcer *et al.*, 1997). After purification, nuclei were subjected to sequential extractions. MA16 was detected in the S2 and NM fractions. Numbers and arrows correspond to the molecular masses in kDa of markers.

different buffers of increasing ionic strength (de Carcer *et al.*, 1997; Medina *et al.*, 1995). First, the initial fraction composed of remnants of the cytoskeleton and proteins of the nuclear envelope (M fraction) was obtained, then a soluble ribonucleoprotein fraction called S2 extract, and a chromatin (Chr) fraction were collected. Finally, two nuclear matrix (NM) fractions were obtained; the high-salt (HS) fraction, and the most insoluble fraction containing proteins associated with a network of RNA filament (NM fraction).

As we show in Figure 2, MA16 is present in the S2 extract and in the last fraction, corresponding to the NM fraction. Interestingly, S2 extracts correspond to a soluble RNP fraction as the low-ionic-strength buffer extracts 70–80% of RNA, and both fibrillarin and nucleolin has been reported to be present in this fraction (de Carcer *et al.*, 1997; Medina *et al.*, 1995). This would be consistent with a role of MA16 in the maturation and/or processing of rRNAs.

Identification of the MA16-interacting protein ZmDRH1

In order to isolate potential MA16-interacting proteins, we used the yeast two-hybrid system to screen a *Z. mays* cDNA library fused to the GAL4 activation domain (AD) in the pGAD vector. A total of 3×10^6 colonies, which had been co-transformed with pAS-MA16, were screened, and independent β -galactosidase-positive clones were analyzed. False positives were eliminated as described in the section under Experimental procedures. Using this approach, we isolated a clone termed ZmDRH1. The MA16-ZmDRH1 interaction was then confirmed by pull-down analysis (see below).

The cDNA insert of ZmDRH1 was about 2.3 kb in length. Sequence analysis revealed that this cDNA encoded a novel protein belonging to the DEAD box RNA helicase protein family. The cDNA encoded a polypeptide of 566 amino acids residues with a calculated molecular mass of 62 kDa (Figure 3a). The protein was named ZmDRH1.

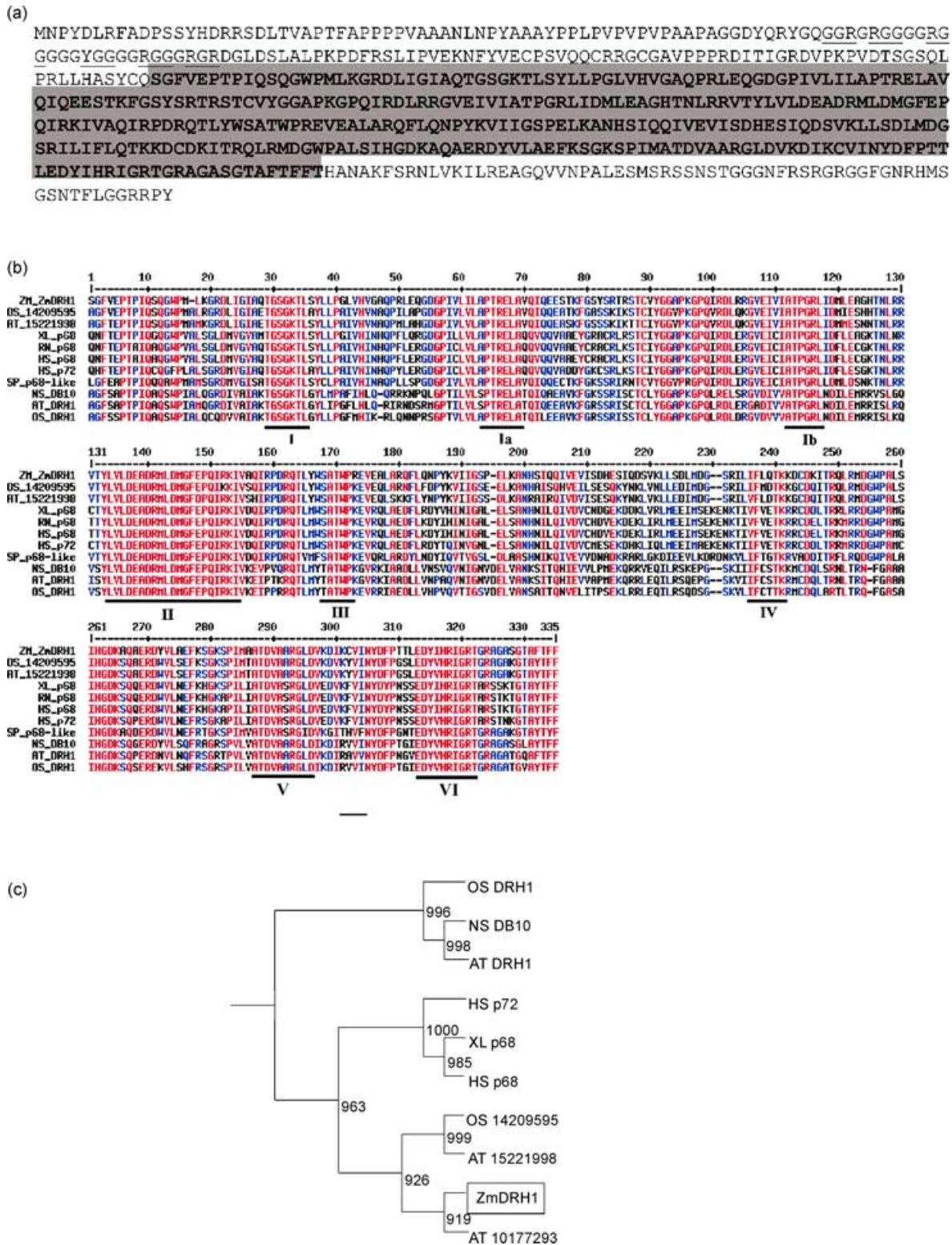


Figure 3. Sequence relationships of ZmDRH1.

(a) Amino acid sequence of ZmDRH1. The gray box indicates the DEAD box helicase domain. RGG motifs are underlined.
 (b) Multiple alignment of the DEAD box helicase domain of ZmDRH1 and close homologs. Numbering from 1 to 335 refers to positions in the alignment. The eight helicase domain motifs are shown.
 (c) Phylogenetic reconstruction using neighbor-joining based on the multiple alignment. Bootstrapping values are shown on the tree. Numbers above 900 indicate high confidence in the clusters. ZM, *Z. mays*; AT, *A. thaliana*; OS, *O. sativa*; NS, *N. sylvestris*; HS, *Homo sapiens*; and XL, *X. laevis*. ATDRH1 is GenBank protein entry 17065030, NS DB10 1169228, OS DRH1 1169228, HS p68 226021, HS p72 5453840, XL p68 8163810.

Southern and Northern blot analyses indicated that a single species of mRNA was produced from a single-copy gene in *Z. mays*.

ZmDRH1 is a basic protein with a calculated pI of 9.43. The ZmDRH1 amino acid deduced sequence can be divided into three distinct domains (Figure 3a). The N-terminal part (1–168) includes a glycine-rich region, which is interrupted by arginine or aromatic residues such as Tyr and Phe and contains three RGG boxes, which suggest that this region is a variant of the RGG motif known to mediate RNA-binding and protein–protein interactions in a number of RNA-binding proteins (Burd and Dreyfuss, 1994). The central region (168–499) harbors the eight highly conserved amino acids motifs characteristic of the DEAD box proteins. Finally, the C-terminal domain (500–566) is rich in glycine (18%) and arginine (13.6%) and includes one RGG box.

ZmDRH1 and other related DEAD box proteins

We next explored in detail the sequence relationships of ZmDRH1 to other DEAD box RNA helicases, by performing BLAST searches (Altschul *et al.*, 1990) against the GenBank database. Remarkable evolutionary proximity was found to a number of plant proteins and to the vertebrate p68/p72 RNA helicase subfamily. Conserved parts in the RNA helicase domain included the eight RNA helicase motifs (Figure 3b). Tree reconstruction by neighbor-joining (Figure 3c) showed that ZmDRH1 was evolutionary closest to a group of plant proteins, including *Oryza sativa* GenBank entry 14209595, and *Arabidopsis thaliana* entries 15221998 and 10177293 (identity level over the RNA helicase domain 78–81%). These proteins are uncharacterized but likely to share functionality with ZmDRH1. A second group of highly related proteins were vertebrate p68 and p72 RNA helicases (64–69% identity). Finally, another subfamily of plant RNA helicases, including *A. thaliana* DRH1 (Okanami *et al.*, 1998), also showed high similarity to ZmDRH1 (56–58% identity). As indicated by the tree topology, these proteins would form a separate cluster to the ZmDRH1 and p68 protein homology group, indicating that they are more distantly related to p68 than ZmDRH1 is.

Although the protein terminal parts are much less conserved than the central helicase domain, many of these related proteins also contain glycine-rich regions with RGG boxes. Among the vertebrate proteins, human p72 and *Xenopus laevis* p68 contain RGG boxes at both the N- and C-terminal regions, while human p68 only at the C-terminus. Among the plant proteins, the pattern is not fully consistent with the evolutionary relationships: RGG boxes are found in the *Arabidopsis* protein, which is most closely related to ZmDRH1 (entry 10177293) but not in the terminal parts of the two other highly related proteins from

Arabidopsis and rice (entries 15221998 and 14209595, respectively), in spite of the fact that RGG boxes can be found at the C-terminal region of the more distantly related group of plant RNA helicases represented by *Arabidopsis* DRH1. These differences in RGG content and the low overall similarity of the terminal parts indicate that these are highly evolving regions. This protein structure may allow for rapid functional diversification, and it illustrates the general view that DBPs contain a similar core region, encompassing the conserved domains, but have N- and C-terminal extensions, which endow the proteins with specialized functions (Aubourg *et al.*, 1999).

Expression of ZmDRH1 mRNA

Northern blot analysis was performed to investigate the expression of ZmDRH1 in different tissues and at different stages of embryogenesis in *Z. mays*. We detected a single band of a transcript of about 2.3 kb in length in total RNA extracted from embryos (Figure 4a). This is in contrast with the observation that in p68 and p72, two transcripts are differently expressed in different tissues (Lamm *et al.*, 1996; Rossler *et al.*, 2000). The expression level was hardly affected by ABA, cold, drought, and wound treatments (Figure 4b). ZmDRH1 is expressed during embryogenesis, with higher levels at the stage of 10–20 days after pollination (DAP). This suggests that ZmDRH1 plays a role during embryo development.

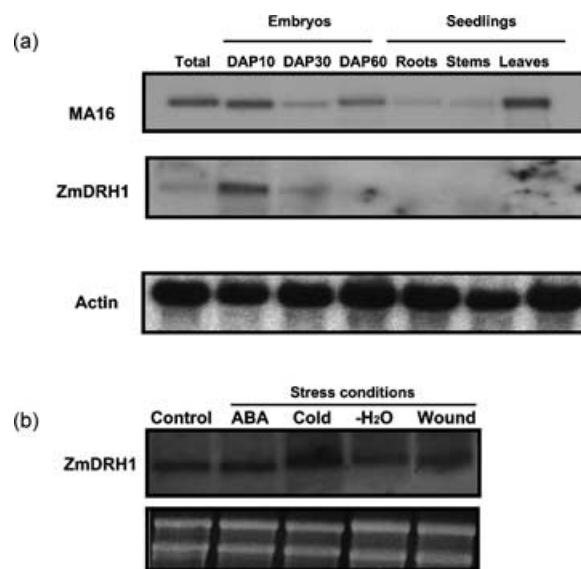


Figure 4. Expression of ZmDRH1 and MA16 during plant development (a) Twenty micrograms of RNA from different tissues was loaded in each lane. Duplicate blots were probed with MA16 and ZmDRH1. Control of RNA loading, probe with actin. (b) ZmDRH1 expression in seedling under different stress conditions: ABA (100 μ M), cold (4°C), desiccation (-H₂O) and wound. A control of RNA loading is shown.

Subcellular localization of ZmDRH1

Most of the DEAD box proteins identified to date are localized in the nucleus (Schmid and Linder, 1992). A computer search using the ZmDRH1 sequence, however, did not reveal any significant homology to nuclear localization sequences (NLS). Therefore, in order to study the subcellular localization of ZmDRH1, the entire coding region of ZmDRH1 was fused to the C-terminus to the GFP reporter gene. The chimeric gene construct and the GFP gene alone were transiently expressed in onion cells by particle bombardment, under the control of the CaMV 35S promoter, and the distribution of the ectopically expressed fusion protein was subsequently examined by immunofluorescence microscopy. We found that ZmDRH1 is localized in the nucleus and accumulated in the nucleolus (Figure 5). In order to identify the determinants responsible for nuclear targeting, a series of deletions were fused to the N-terminus of GFP. Constructs bearing the N-terminal region of ZmDRH1 alone were also fused to GUS-GFP to rule out

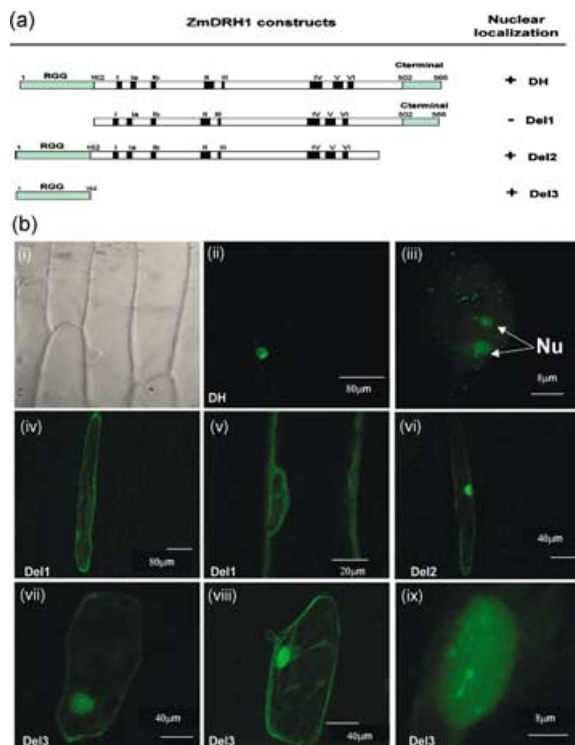


Figure 5. Subcellular localization of ZmDRH1. (a) Schematic representation and nuclear localization of ZmDRH1 deletion mutants. The eight helicase motifs are indicated as black boxes. (b) Representative images of nuclei from transiently transformed onion epidermal cells expressing GFP fusions to portions of ZmDRH1. i–iii, ZmDRH1–GFP; iv and v, Del1–GFP, deletion of the N-terminal domain; vi, Del2–GFP deletion of the C-terminal domain; vii, Del3–GFP construct bearing the N-terminal domain fused to GFP; and viii and ix, Del3–GUS–GFP construct containing only the N-terminal domain fused to GUS–GFP. The transformation was repeated at least three times for each construct.

the possibility that nuclear localization was because of the small size of the fusion protein. This analysis demonstrated that the N-terminal domain, which contains several RGG boxes, is necessary for ZmDRH1 nuclear and nucleolar targeting. The involvement of RGG motifs in nuclear/nucleolar targeting has been recently demonstrated for hnRNPA2 (Nichols *et al.*, 2000), nucleolin (Schmidt-Zachmann and Nigg, 1993), and the nucleolar human RNA helicase II/Gu (RH-II/Gu; Ou *et al.*, 1999). Besides, as shown before, MA16 also requires the RGG region for nucleolar targeting. Our results indicate that the RGG motif is necessary but not sufficient for nucleolar targeting of ZmDRH1. One possibility would be that other ZmDRH1 protein domains are needed as in the case of nucleolin in which the targeting to the nucleolus requires both the RRM and RGG domains.

ZmDRH1 interacts with MA16 through the RRM domain and with fibrillarlin

In order to determine which domain of MA16 interacts with ZmDRH1, we used the two-hybrid system. Full-length MA16 and the RRM and RGG domains were fused to the GAL4–DNA-binding domain (BD). Protein–protein interaction was then determined by monitoring β -galactosidase activity and viability on histidine, adenine, tryptophan, and uracil minus plates. As shown in Figure 6(a,c), we found that

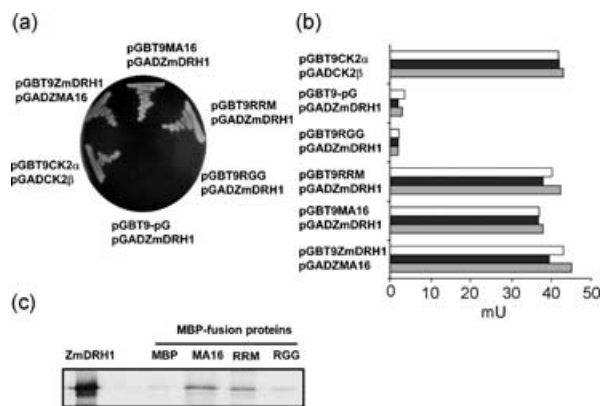


Figure 6. Interaction of MA16 with ZmDRH1. (a) Plasmid pGBT9 containing fibrillarlin, MA16, RRM domain, RGG domain, ZmDRH1 and G protein (negative control) were co-transformed into yeast together with pGAD424 containing ZmDRH1 or MA16. As a positive control, plasmid pGBT9 containing the α -subunit of CKII were co-transformed into yeast with pGAD424 β -subunit of CKII. (b) Graphic representation of β -galactosidase activity measurement in liquid assay from three independent yeast for each combination. Values are expressed as Miller units. (c) *In vitro* pull-down assay of MA16 and ZmDRH1. The *in vitro* translation reaction was carried out in RRL in the presence of 35 S-labeled methionine, and the ZmDRH1 are shown in translation lane (TNT). The 35 S-labeled ZmDRH1 was incubated with resin-bound MBP–MA16, RRM domain, RGG domain, and MBP. After washing with binding buffer, the resin bound proteins were resolved in a 12% SDS polyacrylamide gel.

the interaction with ZmDRH1 is mediated by the MA16 RRM domain.

The interaction was further confirmed by *in vitro* binding assays carried out using maltose binding protein (MBP)-fused MA16, RRM domain, RGG domain, and *in vitro*-translated ZmDRH1 (Figure 6). MBP expressed in *Escherichia coli* BL21(DE3) was used as a negative control. *In vitro* translation of ZmDRH1 was performed in a rabbit reticulocyte lysate (RRL) using RNA transcripts generated by T7 RNA polymerase. ³⁵S-labeled ZmDRH1 of the expected size was produced and appeared as a major band in the *in vitro* translation. The *in vitro* pull-down results were consistent with the two-hybrid system results, confirming that MA16 interacts directly with ZmDRH1 through the RRM domain in a specific manner. The association of ZmDRH1 and MA16 is unlikely to be mediated by RNA because RNase treatment did not affect the binding of the two proteins.

As ZmDRH1 is found in nuclei, accumulates in nucleolus, and shows a high similarity to p68, which has recently been reported to bind to fibrillarin, we decided to investigate whether ZmDRH1 was able to interact with fibrillarin. Full-length fibrillarin was fused to GAL4-DNA-BD and used for yeast two-hybrid analysis. As shown in Figure 7, ZmDRH1 binds to fibrillarin. Using the same technique, a direct interaction between MA16 and fibrillarin could not be detected. To confirm the *in vitro*-specific binding, we employed pull-down assays with glutathione-S-transferase (GST)-fibrillarin fusion protein and *in vitro*-translated [³⁵S]methionine-labeled ZmDRH1. The results obtained were coherent with the yeast two-hybrid experiments. Taken together, these data suggest that MA16, ZmDRH1,

and fibrillarin may be part of a type of ribonucleoprotein complex involved in ribosomal biogenesis.

Discussion

Our previous data suggest that the maize RNA-binding protein MA16 may be forming part of an RNP. In the present study, we have identified and characterized a novel DEAD box RNA helicase, named ZmDRH1, that interacts with MA16. ZmDRH1 is a 62-kDa nucleolar protein from *Z. mays* closely related to ATP-dependent DEAD box RNA helicases, enzymes that unwind nucleic acid duplexes. DEAD box proteins are characterized by the presence of eight conserved motifs (Tanner and Linder, 2001), grouped in a central core domain of about 300–350 amino acids. Despite their sequence conservation, DEAD box RNA helicases differ mainly by the addition of N- and C- terminal sequences, which vary in length and composition and are believed to be involved in conferring substrate specificity (Tsu *et al.*, 2001) and/or binding accessory proteins (de la Cruz *et al.*, 1999). They are also important in the regulation of the intracellular distribution of the protein as it was found for Prp22 and Prp16, in which the N-terminal region is responsible for localization in spliceosomes (Schneider and Schwer, 2001).

The DEAD box RNA helicases are found in several species ranging from bacteria to mammals, and they are involved in almost all cellular processes involving RNA molecules (Linder and Daugeron, 2000). They are also important cellular factors for regulatory events, in particular, during organ maturation and cell growth and differentiation (Jacobsen *et al.*, 1999; Nichols *et al.*, 2000)

Database search showed a very close relationship of ZmDRH1 to p68 DBPs. p68-type DBPs have been found in a variety of eukaryotes, including yeast (DBP2; Iggo and Lane, 1989), *Drosophila* (RM62; Dorer *et al.*, 1990), mouse (p68; Lemaire and Heinlein, 1993), human (p72 and p68; Iggo *et al.*, 1991; Lamm *et al.*, 1996), tobacco (DB10; Itadani *et al.*, 1994) and *Arabidopsis* (AtrH1; Okanami *et al.*, 1998).

Here, we have identified several additional plant RNA helicases, which are highly related to ZmDRH1 and vertebrate p68 proteins. Several functions have been assigned to p68 and p72 such as transcriptional co-activation of estrogen receptor α (Watanabe *et al.*, 2001), pre-mRNA splicing (Honig *et al.*, 2002; Liu, 2002), development and differentiation (Kitamura *et al.*, 2001), and tumor development (Causevic *et al.*, 2001). In fact, a recent publication has shown that p68 can exist in a variety of complexes, which would be consistent with the wide range of functions for p68/p72 (Ogilvie *et al.*, 2003). Interestingly, depletion of Dbp2p in yeast results in defects in both nonsense-mediated mRNA decay and rRNA processing and expression of p68 in *dbp2* mutants could reverse the rRNA

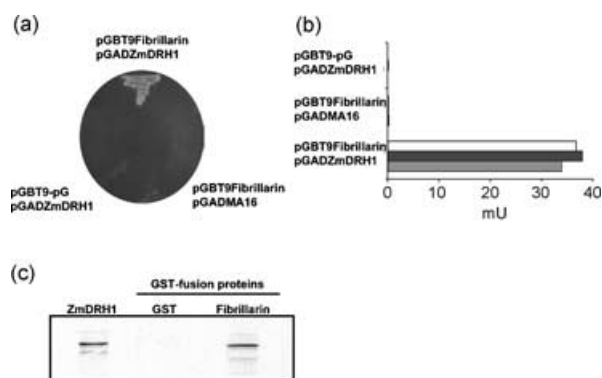


Figure 7. Interaction of fibrillarin with ZmDRH1 and MA16.

(a) Interactions of fibrillarin with ZmDRH1 and MA16. Plasmid pGAD424 containing MA16, ZmDRH1, and G protein (negative control) were co-transformed into yeast together with pGBT9 containing fibrillarin.

(b) Graphic representation of β -galactosidase activity measurement in liquid assay from three independent yeast for each combination. Values are expressed as Miller units.

(c) *In vitro* pull-down assay of fibrillarin and ZmDRH1. Proteins were resolved in a 12% SDS polyacrylamide gel.

processing defect, suggesting that p68 could play a role in rRNA processing in human cells (Bond *et al.*, 2001).

All eukaryotic DEAD box proteins identified to date are localized within the nucleus (Schmid and Linder, 1992) with the exception of eIF-4A (Linder *et al.*, 1989), Suv3p (Margossian *et al.*, 1996), and MSS116 (Seraphin *et al.*, 1989), which are found in the cytoplasm. Only four proteins, human RH-II/Gu (Valdez *et al.*, 1995), the human NOH1 (Zirwes *et al.*, 2000), yeast Rok1p (Venema *et al.*, 1997), and yeast Dbp3p (Weaver *et al.*, 1997) have been found to accumulate in the nucleolus. Moreover, the human p68 (Iggo *et al.*, 1991) and the *Xenopus* protein An3 (Gururajan and Weeks, 1997) have been reported to be transiently associated with the nucleolus in either a cell cycle or a development-specific manner. Here, we show that ZmDRH1 is a nuclear-localized protein, which is also present in the nucleolus. In order to define the region(s) necessary for this targeting, we have analyzed various deletion constructs fused to the GFP. Interestingly, the N-terminal 120-amino-acid region, which contains the RGG domain and some flanking amino acids, was necessary and sufficient to direct GUS/GFP to the nucleus. The involvement of the RGG domain in subcellular localization has been recently demonstrated for several proteins. Our results showed that, in the case of MA16, the RGG domain is also necessary and sufficient for nuclear and nucleolar targeting. Deletion of RGG domains in nucleolin (Heine *et al.*, 1993) and fibrillarin (Pih *et al.*, 2000) resulted in altered localization to the nucleolus. All these findings are in agreement with the hypothesis, supported by our results, that the RGG domain functions to promote nuclear/nucleolar localization. Apart from being a subcellular localization determinant, the RGG domain has been proposed to serve functions as diverse as RNA binding (Kiledjian and Dreyfuss, 1992) or/and protein–protein interactions (Bouvet *et al.*, 1998). It is interesting to note that frequently the RGG domain contains the modified amino acid N^G - N^G -dymethyl-arginine. In fact, RGG-box-containing peptides derived from nucleolin, fibrillarin, and hnRNPU are substrates for this modification, and probably this could serve to modulate the RNA-binding and/or protein–protein interactions (Raman *et al.*, 2001).

Our studies demonstrate that ZmDRH1 is able to interact directly with MA16 *in vitro*. This interaction is mediated by the RRM, suggesting that, as it happens in the case of the C-terminal RRM of U2AF (Selenko *et al.*, 2003) or *Drosophila* Y14 RRM domain (Shi and Xu, 2003), this region may also act as a protein interaction domain. Interestingly, in our nuclear fractionation experiment, MA16 is present in both S2 extract and the NM fraction. According to published data for both animal and plant cells, S2 extract is a soluble RNP fraction and contains different nucleolar proteins such as the CKII, fibrillarin, and nucleolin (de Carcer *et al.*, 1997; Medina *et al.*, 1995). Recently, it was found that p68

co-immunoprecipitated *in vivo* with fibrillarin (Nicol *et al.*, 2000); here, we also observed an association between ZmDRH1 and fibrillarin. Fibrillarin has been shown to be essential for pre-rRNA processing, pre-rRNA methylation, and ribosome assembly (Barneche *et al.*, 2000; Tollervey *et al.*, 1993). We have previously reported that MA16 is localized in nuclei, preferentially accumulated in the dense fibrillar component (DFC), where it is believed that the processing of pre-rRNA takes place (Alba *et al.*, 1994). On the other hand, fibrillarin might be involved in additional activities such as splicing and tRNA processing (Bertrand *et al.*, 1998).

Therefore, MA16 could be involved in processing of pre-rRNA and/or ribosomal biogenesis, splicing, and/or tRNA. In all these processes, a multitude of RNA–RNA, RNA–DNA, and RNA–protein interactions must transiently form and dissociate in a specific order and in an efficient manner (Kramer, 1996). DEAD box RNA helicases could play a crucial role in all these processes, both in structural rearrangements and as directional driving forces. Moreover, RNA helicases are often found within large ribonucleoprotein complexes, e.g. translation initiation, ribosomal precursors, and spliceosomes (Tanner and Linder, 2001). Thus, taken together, these results suggest that MA16, ZmDRH1, and fibrillarin may be forming part of a nucleolar RNP complex, and/or MA16 is an essential RNP-multifunctional protein component serving different functions in the cell.

An important goal for future studies will be to identify the *in vivo* RNA substrates and proteins involved in such RNP complexes, which should allow decoding of their function more precisely.

Experimental procedures

Plant material

Embryos of maize (*Z. mays*) pure inbred line W64A were collected before pollination (BP) and at 10, 20, 30, and 40 DAP. Maize seedlings grown for 7 days were used to obtain leaf, root, and stem tissues. Water-stress, wound, cold, and ABA treatments of maize seedlings were performed as described previously by Gomez *et al.* (1988).

Purification of nuclei

To obtain nuclei from *Z. mays* and *Arabidopsis* cells, we used the method modified from Hamilton *et al.* (1972). For nuclei isolation, about 50–100 g of tissue was harvested. After adding ice-cold diethylether, the tissue was washed with cold water. Then, the tissue was incubated with extraction buffer (1 M sucrose, 25 mM Tris–HCl, 10 mM MgCl₂, 0.2% Triton, and 7 mM β-mercaptoethanol), homogenized in a high-speed blender, and filtered. After centrifugation the pellet was re-suspended in buffer A (extraction buffer plus 0.5% Triton). After layered over two discontinuous

Percoll gradients (60% (v/v) and 35% (v/v) percoll:bufferA), and centrifugation, the nuclei appeared in a band corresponding to 35–65% interface. The nuclei were purified and finally re-suspended in nuclei stock buffer (NSB: 10 mM Tris-HCl, 10 mM HEPES, 2 mM MgCl₂, 10 mM KCl, 4 mM *n*-octanol, 0.1 mM CaCl₂, pH 7.4). A cocktail of protease inhibitors, consisting of 1 µg ml⁻¹ aprotinin, 1 µg ml⁻¹ leupeptin, 1 µg ml⁻¹ pepstatin, and 0.1 mM phenylmethylsulfonylfluoride (PMSF) from Sigma (St Louis, MO, USA) was added to all buffers.

Nuclear fractionation

The procedure was based on the method described by de Carcer *et al.*, (1997). Isolated nuclei were first treated with 1% (v/v) Nonidet P-40 and 0.5% (v/v) sodium deoxycholate in NSB. After centrifugation for 10 min at 4°C, nuclei were extracted for 1 h at 4°C, with a low-ionic strength buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The pellet was treated with 0.1 mg ml⁻¹ of RNase-free DNase in NSB for 30 min at room temperature (RT), and the digested DNA was extracted with 0.25 M ammonium sulphate, in NSB, for 5 min at RT. Finally, the pellet was re-suspended in NSB and a similar volume of 4 M NaCl was added dropwise with gentle shaking to obtain a final concentration of 2 M NaCl. The insoluble pellet corresponds to the NM. Fractions were characterized using different antibodies such as antibodies against histones.

Northern analysis

For Northern blot analysis, total RNA was prepared from roots, stems, leaves, and embryos at different stages of development by phenol extraction as previously described by Busk and Pages (1997). For stress experiments, RNA was isolated from seedlings exposed to cold (4°C), wound, desiccation, and ABA (100 µM). RNA blotting was performed essentially as described by Sambrook *et al.*, (1989). Briefly, 20 µg of total RNA was fractionated in a formaldehyde-containing 1% agarose gel, stained with ethidium bromide, and transferred onto a Hybond-N filter (Amersham Bioscience, Bucks., UK). The *EcoRI*-*XhoI* fragment of ZmDRH1 was radiolabeled by random priming and used to hybridize the filters. Hybridization and washing of the filters were performed under stringent conditions.

Yeast two-hybrid screening

A HybridZap two-hybrid vector system library was constructed from poly(A)⁺ RNA from stressed leaves of the maize inbred line W64A, according to the manufacturer (Matchmaker, Clontech Laboratories Inc., Palo Alto, CA, USA). MA16 cDNA was cloned in pAS2 in frame to produce a GAL4-DNA-BD fusion protein. The yeast strain AH109 was transformed with the pAS-MA16 DNA and assayed for β-galactosidase activity to confirm that MA16 did not have autonomous transactivating activity. The pAS-MA16 AH109 strain was then transformed with the library fused to the GAL4 AD in pGAD4 vector. A total of 6 × 10⁶ colonies were screened and positive clones were selected in Leu, Trp, Ade, and His plates and tested for β-galactosidase activity using filter assays according to the manufacturer (Matchmaker, Clontech Laboratories Inc.). Plasmids from positive clones were isolated and the DNA sequence of the inserts was determined. False positives were eliminated by co-transforming putative positive clones with a negative control (pAS-proteinG and pAS alone) and a positive control (pAS-MA16).

Yeast two-hybrid interaction testing

To generate the GAL4 BD fusion proteins, the cDNAs encoding MA16, RRM domain, RRG domain, G protein, fibrillarin, ZmDRH1, and CKII α were cloned into pGBT9 vectors. To obtain pGBT9-fibrillarin, the cDNA from *Arabidopsis* was cut with *NcoI* and cloned into the pGBT9 vector. Fusion proteins with GAL4 AD were obtained cloning the cDNAs encoding ZmDRH1, CKII β, and MA16 into pGAD vector. pGAD-ZmDRH1 was isolated in the two-hybrid library screening. For interaction studies, both plasmids containing chimeric proteins fused to AD and BD were co-transformed into *Saccharomyces cerevisiae* AH109 strain. Clones were selected in Leu, His, Ade, and Trp plates and tested for β-galactosidase activity using filter and liquid assays according to manufacturer.

In vitro binding experiments

MA16 and the different domains were cloned in the pMAL expression vector (Biolabs). Fibrillarin was cloned into pZEX vector (Jimenez *et al.*, 1997) Expression of MAL-GST fusion proteins and binding assays were performed as described previously (Jimenez *et al.*, 1997). ZmDRH1 was translated in the presence of [³⁵S]methionine (>1000 mCi mmol ± 1, Amersham), using the T7-TNT Quick coupled Transcription/Translation System (Promega Corporation, Madison, WI, USA). For *in vitro* binding assays, 2–3 µg of each MBP fusion protein and fibrillarin-GST fusion protein was attached to 30 µl of beads and incubated with 2 µl ³⁵S-labeled protein in 180 µl of binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 2.5 mM MgCl₂, 10% glycerol, 0.2% NP-40, 1 mM DTT, and 3 µl of rabbit non-immune serum). After incubation overnight at 4°C by shaking, the beads were washed four times with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.2% NP-40), re-suspended in 20 µl of electrophoresis sample buffer, boiled for 5 min, and separated by 12% SDS-PAGE. The radiolabeled proteins were then visualized by autoradiography.

Subcellular localization experiment with GFP fusion

Standard molecular cloning procedures (Sambrook *et al.*, 1989) were employed, and details of the clone construction are available upon request. The binary vector pCAMBIA-1302 (Hajdukiewicz *et al.*, 1994) contains a dual 35S promoter from CaMV, the translational leader sequence from tobacco etch virus (TEV), and the 35S polyadenylation signal from CaMV and mGFP5 (Haseloff *et al.*, 1997), and therefore expresses correctly spliced transcripts in *Arabidopsis*. The cloning of N-terminal fusions to GFP or GUS-GFP via *NcoI* and *SpeI* sites. Fusion protein genes were constructed based on the full-length ZmDRH1 and MA16 cDNA sequence using oligonucleotides to introduce suitable restriction enzyme recognition sites. PCR amplification of cDNAs was carried out with high-fidelity DNA polymerases. The DNA sequence of pCAMBIA 1302/1303 are available under GenBank Accession number AF234298/99. Transient expression assays in onion epidermal cells using particle bombardment (Bio-Rad, Richmond, CA, USA) were carried out as described previously by von Arnim and Deng (1994). Tissues were examined after overnight incubation at 22°C in the dark using confocal laser scanning microscopy (Leica TCS SP laser scanning confocal microscope).

Sequence analysis

We performed BLAST searches (Altschul *et al.*, 1990) against the GenBank database to identify homologs of ZmDRH1. The DEAD

box RNA helicase domain of a selected group of proteins, corresponding to the closest homologs, was aligned using MULTIALIN (Corpet, 1988). Phylogenetic tree reconstruction by neighbor-joining was performed with CLUSTALW (Thompson *et al.*, 1994). One thousand bootstrapping cycles were performed to assess confidence in the clusters. The tree was rooted with the distantly related DBP5 from human and mouse (GenBank protein entry 5701850 and 26986593, respectively). For tree visualization TREEVIEW (Page, 1996) was used. The amino acid composition and the molecular weight of ZmDRH was determined using the PEPTIDESORT program (Devereux *et al.*, 1984).

Nucleotide sequence accession numbers

Sequences of the DEAD box RNA helicase from *Z. mays* and fibrillarlin from *A. thaliana* genes are deposited in the GenBank nucleotide sequence database under Accession numbers AY466159 and X99938.

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