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Two DEAD-box proteins may be part of RNA-dependent high molecular weight protein complexes in *Arabidopsis thaliana* mitochondria

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ABSTRACT

Posttranscriptional processes are important for regulation of gene expression in plant mitochondria. DEAD-box proteins, which form a huge protein family with members from all kingdoms, are fundamental components in virtually all types of processes in the RNA metabolism. Two members of this protein family designated PMH1 and PMH2 (for putative mitochondrial RNA helicase) were analyzed and characterized in mitochondria of *Arabidopsis thaliana*. GFP tagging with N-terminal PMH1 and 2 sequences supports the mitochondrial localization of these proteins. Northern experiments as well as histochemical glucuronidase (GUS) staining of transgenic plants carrying respective promoter:GUS fusion constructs revealed differing transcription patterns for the two genes. In response to cold, however, transcript levels of both genes increased. Immunodetection analyses of mitochondrial protein complexes after two-dimensional blue native/urea sodium dodecyl sulfate polyacrylamide gel electrophoresis and after fractionation on sucrose gradients strongly suggest that one or both proteins are part of RNA-dependent complexes. Cold treatment of cell cultures or solubilization of mitochondria in the presence of $MgCl_2$ favored the detection of high molecular weight complexes. This study paves the way for a detailed analysis of high molecular weight complexes in mitochondria of higher plants.

INTRODUCTION

Various posttranscriptional processes so far described in plant mitochondria have been suggested to be important for regulation of gene expression in this subcellular compartment (Hoffmann and Binder, 2002; Binder and Brennicke, 2003; Marchfelder and Binder, 2004; Gagliardi and Binder, 2007). This includes intron splicing, RNA editing, trimming of 3' ends and processing at the 5' termini of rRNAs, tRNAs and mRNAs and may also apply for regulatory processes at the level of translation. Although considerable progress has been made

in elucidating the proteins involved in some of these processes, the (vast) majority of these components are still unknown. Purification of such proteins has been found to be difficult or in many cases impossible, mostly due to the low abundances of these proteins but also due to limitations in respective *in vitro* systems, which are required to investigate the actual participation of a purified protein in a certain process. Alternatively genetic approaches can be used to identify genes whose products are involved in RNA processing in plant mitochondria. For instance, forward genetics searching for *rf* (restorer of fertility) genes restoring cytoplasmic male sterility in different plant species identified several PPR proteins, directly or indirectly involved in degradation, stability determination or cleavage of CMS-associated transcripts (Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Kazama and Toriyama, 2003; Koizuka et al., 2003; Wang et al., 2006). Reverse genetic approaches have been successfully applied to assign functions to *in silico* identified proteins as for instance to a mitochondrial PNPase and an RNase II-like protein called RNR1, respectively (Perrin et al., 2004a; Perrin et al., 2004b). These proteins are involved in mRNA 3' processing and RNA degradation as well as in processing of ribosomal RNA.

DEAD-box proteins form a large gene family and are present in almost all organisms from all kingdoms (Linder, 2006). In *Arabidopsis thaliana* 58 genes for DEAD-box proteins have been identified (Boudet et al., 2001; Mingam et al., 2004). Most of these proteins are considered to be RNA helicases, although this particular function was actually analyzed for only a few of these proteins (Rocak and Linder, 2004). The primary function of DEAD-box RNA helicases is to rearrange inter- or intramolecular RNA structures or to dissolve RNA-protein complexes (Rocak and Linder, 2004; Linder, 2006). These actions, dependent on ATP hydrolysis, are often prerequisites for subsequent processing steps and RNA helicases are often associated with other proteins. Consequently DEAD-box proteins have been implicated to participate in all kind of processes dealing with RNA including RNA synthesis, RNA modification, RNA cleavage, RNA degradation as well as ribosome biogenesis and translation

initiation (Linder, 2006). DEAD-box proteins can thus be ideal starting points for the detection and purification of multiprotein complexes involved in gene expression in plant mitochondria. In addition DEAD-box proteins and their genes can be readily identified by their typical conserved motifs (Rocak and Linder, 2004; Linder, 2006).

To identify proteins involved in the RNA metabolism in higher plant mitochondria we have now analyzed two DEAD-box proteins in *Arabidopsis thaliana*. We determined their subcellular localization and investigated transcript levels as well as the promoter activities of these two genes. We find that these proteins are integrated in RNA-dependent high molecular weight complexes.

RESULTS

Posttranscriptional processes play a key role in gene expression in plant mitochondria (Gagliardi and Binder, 2007). To date still little is known about the components involved in the individual RNA maturation steps. To get hold of such proteins in plant mitochondria we started to examine DEAD-box proteins in *Arabidopsis thaliana*. The genes and gene products for these studies, At3g22310 and At3g22330, were described in previous global *in silico* studies of this class of proteins in this plant model species (Aubourg et al., 1999) and were detected in proteomic investigations of mitochondria (Millar et al., 2001; Heazlewood et al., 2004). The latter and our own *in silico* analyses (data not shown) indicated these proteins to be targeted to mitochondria suggesting them to be good candidates for studying components of the mitochondrial RNA metabolism. These proteins, designated putative mitochondrial RNA helicase 1 (PMH1, At3g22310) and 2 (PMH2, At3g22330), contain almost all motifs typical for DEAD-box proteins (Supplementary Fig. 1) (Rocak and Linder, 2004), with the exception of motif IV. PMH1 and 2 are very similar to each other with 77 % identical amino acids, both carrying a Ser/Gly-rich C-terminus. As seen from the AGI numbers the genes are

encoded on chromosome III, only separated by the gene (At3g22320) encoding the 24.3 kDa subunit of RNA polymerases I to III (Arabidopsis Genome Initiative, 2000).

Subcellular localization of PMH1 and 2

PMH2 has previously been found in proteome analyses of plant mitochondria indicating that this protein is located in this organelle (Millar et al., 2001; Heazlewood et al., 2004). In addition *in silico* prediction of subcellular targeting by prediction programs TargetP (http://www.cbs.dtu.dk/services/TargetP/) and Predotar (http://urgi.infobiogen.fr/predotar/predotar.html) strongly suggest a mitochondrial localization of both PMH1 and 2. To address this question experimentally two cDNA fragments of each gene (corresponding to 69 and 100 amino acids of PMH1 and 62 and 104 amino acids of PMH2, respectively) were fused upstream in frame to the reading frame encoding the green fluorescent protein (GFP) (Davis and Vierstra, 1998). The constructs were transformed into tobacco protoplasts stained with MitoTracker Red, a dye that specifically accumulates in mitochondria. The subcellular localization of the fusion proteins was monitored by fluorescent microscopy. Co-localization of the green fluorescence of all four fusion proteins with the fluorescence of MitoTracker Red revealed that the PMH:GFP fusion proteins are imported into these organelles (Fig. 1, data not shown). This strongly suggests that also *in vivo* PMH1 and 2 are targeted to mitochondria, which is further substantiated by the detection of these proteins in mitochondrial lysates (see below and references (Millar et al., 2001; Heazlewood et al., 2004).

PMH1 and 2 show differing expression patterns but are both induced by cold treatment

The high similarity of these proteins suggests that they might have similar functions, which can complement each other. As a first step we investigated if they are expressed in different tissues and developmental stages. In northern analyses, performed with gene-specific probes

covering the 5' untranslated regions (UTRs) as well as the unique regions of the mitochondrial targeting sequences, *PMH1* steady state transcripts are mainly found in flowers, at very low levels in roots and seedlings but are hardly detectable in leaves (Fig. 2, left panel). Since many RNA helicases from bacteria and various eukaryotic organisms show an enhanced expression at low temperatures (Owttrim, 2006), we also analyzed *PMH1* mRNA levels in seedlings grown at 4° C. A strong signal in the northern analysis demonstrates a high expression of *PMH1* under these conditions. The analogous northern experiment performed with the *PMH2* probe also detected a predominant transcription of this gene in flowers (about three-fold higher than in seedlings), but revealed also *PMH2* transcripts in roots, seedlings and leaves (Fig. 2, right panel). Similar to *PMH1* an elevated amount of *PMH2* mRNAs was found after cultivation of seedlings for 24 h in the cold (approximately 2.7-fold higher), although induction seems to be less strong (Fig. 2, right panel).

The enhanced *PMH1* transcription after cold treatment was further analyzed and quantitatively resolved over time. An about seven-fold induction is seen already 12 h after the transfer of the seedlings to 4°C. A three-fold increased mRNA level is still detectable after 48 h. Maximal induction is observed after 36 h with an approximately ten-fold higher amount of *PMH1* mRNAs in comparison to the control plants grown under identical conditions at 23°C (data not shown).

Transcriptional activity was studied in more detail using the β-glucuronidase (GUS) reporter gene. To this end 2.0 kb upstream of the ATG containing potential promoter sequences of both genes were fused to the GUS reading frame. This region upstream of PMH1 includes the preceding gene At3g22300 and 331 bp of its promoter. The PMH2 upstream region comprises the gene At3g22320, which is encoded on the opposite strand.

After transformation of the respective constructs into *A. thaliana*, five different plant lines originating from five individual integration events per construct were examined by histochemical GUS staining. Substantial staining is consistently observed in transgenic plants

expressing GUS under the control of the PMH2 promoter. Strong promoter activities are indicated in cotyledons, in emerging leaves, in expanded leaves as well as in root tips of six, nine and 16 day-old seedlings grown on MS medium (Fig. 3) or soil (data not shown). Transcriptional activity is also observed in roots, in anthers and in the basal parts of siliques of adult plants. In addition pronounced staining is found in tissue parts close to the cut sites of detached leaves and siliques as well as of parts of flowering stalks suggesting that transcription of *PMH2* is triggered by wounding (Fig. 3). This is confirmed by experiments in which the leaf blades were mechanically wounded by a cut or a pinprick. All of these mechanical injuries consistently trigger promoter activity in tissues adjacent to the site of treatment (Supplementary Fig. 2).

A different pattern of activity was found in the plants expressing GUS governed by the *PMH1* promoter. While no staining at all is detectable in seedlings grown on soil (data not shown), a very weak promoter activity is indicated in the root tips of seedlings cultivated on MS medium under otherwise identical conditions (Fig. 3 upper part, indicated by an arrow). GUS activity is not seen in any part of the adult plants except a substantial promoter activity restricted to anthers. In contrast to *PMH2* no induction by mechanical treatment is observed for *PMH1* indicating that both genes respond differently to this external stimulus. All in all the results of the northern analysis are consistent with the observations in the histochemical analyses, both indicating different expression patterns for each gene suggesting that the two proteins have partially different roles in the plant.

Both PMH proteins are expressed in *A. thaliana* cell suspension culture

For further analysis of the PMH proteins two PMH1-derived peptides were synthesized and used for antibody production in rabbits (Supplementary Fig. 1). The antiserum should detect both proteins, and indeed also binds to recombinant PMH2 (data not shown).

To investigate whether both proteins are expressed in an *A. thaliana* cell suspension culture, mitochondria were isolated and purified from this tissue. Proteins from these organelles were separated by isoelectric focusing (IEF) on immobilized nonlinear pH gradients ranging from pH 3 to 11 followed by standard SDS PAGE. Staining with Coomassie blue revealed a protein pattern similar to those observed in previous proteome analyses of mitochondria (Kruft et al., 2001). Several weak spots corresponding to proteins of 60 kDa are present between pH 9 and 10, where PMH2 has been found previously (Fig. 4A, highlighted by a dashed box) (Millar et al., 2001). Proteins in this range were then inspected by immunodetection with the PMH1/2 antiserum. A single series of spots is observed exactly at the expected position corresponding to proteins of about 60 kDa with a pI between pH 9 and 10 (Fig. 4B). To obtain a higher resolution in this range, mitochondrial proteins were separated on a gradient spanning pH 7 to 11. This revealed two rows of protein spots in the expected area in the stained gel (indicated by arrows a and b in Fig. 4C). Immunodetection analysis of such a gel detected at least five different spots, which correspond to the upper row (Fig. 4D). To unambiguously identify the proteins detected by the PMH1/2 antiserum, spots of both rows were analyzed by mass spectrometry (indicated by open rectangles in Fig. 4C). In spots one to four of the upper row predominantly peptide masses matching fragments specific for PMH1 or 2 were found, while spot five contains only peptides corresponding to PMH2. In the spots of the lower row peptides predominantly matching to translation elongation factors (At1g07920, At1g07930, At1g07940) were identified. These proteins have also been found previously in the mitochondrial proteome of *A. thaliana* (Heazlewood et al., 2004).

Taken together this analysis shows that PMH1 as well as PMH2 are expressed in this *A. thaliana* cell suspension culture. In addition these experiments confirm that the antiserum recognizes the PMH proteins and can be used for selective detection of these proteins in mitochondrial lysates.

Detection of PMH1 and/or 2 in RNA-containing polypeptide complexes

The PMH1/2 antiserum was then used to examine whether these DEAD-box proteins are associated with other proteins. To follow this issue, 1 mg of total mitochondrial protein isolated from the cell suspension culture was solubilized with dodecylmaltoside (DDM) and separated by two-dimensional blue native (BN)/urea sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Coomassie staining of proteins after the separation revealed a pattern, in which distinct complexes can be identified by comparison with the results of analogous separations (Fig. 5A) (Eubel et al., 2003). An immunodetection analysis of analogously separated proteins with the PMH1/2 antibody revealed protein complexes with apparent sizes ranging between less than 100 kDa and up to 1,500 kDa (Fig. 5B). The detected proteins or complexes do not correspond to any of the highly abundant protein complexes visible on the Coomassie stained gel (Fig. 5A and B). The signal corresponding to a protein size of less than 100 kDa most likely represents monomeric PMH1 or 2 or a mixture of both proteins. In addition, a slightly larger protein can be seen, which is also detected in the first and second fractions of the sucrose gradient (indicated by an arrow in Fig. 5B and D, see below). Furthermore a single protein with an apparent molecular weight of 30 kDa also cross-reacts with the PMH1/2 antiserum.

DEAD-box proteins are involved in multiple processes that require interaction of these proteins with RNA. We thus tested whether the integrity of the PMH1/2 containing complexes depends on the presence of RNA. To this end solubilized total mitochondrial protein was treated with RNase A prior to separation by two-dimensional BN/urea SDS PAGE. Digestion of the RNA almost completely disassembled the high molecular weight complexes (Fig. 5C). Beside a weak signal corresponding to a size of about 580 kDa (ATP Synthase) only the signal most likely representing the monomeric protein can now be detected (Fig. 5C). This strongly suggests that the stability or maintenance of the detected complexes or at least the association of PMH1 or 2 with the complexes depends on the presence of RNA.

Since cold treatment enhanced the steady state transcript levels of both PMH proteins we also examined the influence of cold on complex composition and/or size. The cell suspension culture was grown under normal conditions and then incubated for 18 h at 4°C under otherwise identical conditions. Mitochondria were isolated and proteins were investigated as in the previous experiments. This treatment decreased the abundance of the complexes with sizes below 480 kDa (corresponding to the size of complex III; Fig. 5D). In contrast substantially more complexes are detectable that are even larger than those observed in mitochondria from normally grown cultures (Fig. 5D). A similar complex pattern is apparent when the mitochondrial protein is solubilized in the presence of 10 mM $MgCl_2$, which can stabilize ribonucleoprotein particles (Fig. 5E). Again a shift towards higher molecular masses is seen under these conditions.

In summary our analysis suggests that PMH1 and/or 2 are part of RNA-dependent high molecular weight complexes. The size of the protein complexes itself or at least the interaction of the PMH proteins with complexes of higher molecular masses increases upon cultivation of the cells at 4°C or by the presence of $MgCl_2$ during solubilization.

PMH 1 and/or 2 are associated with a very large complex

To investigate the complex association of the PMH proteins by an independent experimental procedure, DDM-solubilized mitochondrial protein was fractionated in discontinuous sucrose gradients. In addition this experiment will allow conclusions about a potential interaction of the PMH proteins with ribosomes. Thus 100 mg of mitochondria were solubilized with DDM under conditions optimized for the enrichment of intact mitochondrial ribosomes and polysomes (Raczynska et al., 2006). The lysate was size fractionated on a sucrose step gradient with steps of 5 % ranging from 15 to 55 % (w/v) sucrose, each step was collected separately and proteins and RNA were analyzed.

The proteins were investigated by an immunodetection analysis with the PMH1/2 antiserum after SDS PAGE of 50 µl of each fraction. PMH1 and/or 2 are detected in fractions one to seven and a slightly larger protein is found mainly in fractions one and two (Fig. 6A, upper part). This protein has also been detected in BN/SDS PAGE, but had not been seen in the IEF/SDS PAGE analysis, which might be attributed to conditions during IEF.

To determine the localization of ribosomes or (polysomes) in the gradient total RNAs were extracted from 800 µl of each sucrose gradient fraction and inspected by agarose gel electrophoresis. The rRNAs were found predominantly in fractions three to five but only found in minor amounts in fraction two, where the PMH proteins are highly abundant (Fig. 6A). This experiment demonstrates that a large portion of the PMH proteins does not co-fractionate with ribosomes. When mitochondria are lysed in the presence of 300 mM KCl (Fig. 6B), the ribosomal RNAs are again found in the same fractions indicating that they remained intact as expected from a previous analysis (Fig 6B, upper part) (Raczynska et al., 2006). In contrast, most of the PMH proteins are now found in fractions one and two indicating that they are released from their complexes (Fig. 6B, bottom part). These experiments strongly suggest that the PMH proteins are not integrated into or stably associated with ribosomes, but we can presently not exclude a weak or transient interaction of the PMH proteins with the translation machinery.

DISCUSSION

PMH1 and 2 are mitochondrial proteins

In this study we analyze two DEAD-box proteins encoded in the nuclear genome of *Arabidopsis thaliana*. We provide evidence for the localization of the two proteins in mitochondria (Fig. 1). Fusion proteins consisting of different N-terminal parts of these polypeptides and the green fluorescent protein were consistently transported into these organelles. In addition the putative RNA helicases were repeatedly detected in mitochondrial

protein fractions (Fig. 4 to 6). This co-fractionation has been observed previously in proteomic analyses applying gel-based and LC-based separations all supporting the localization of the two proteins in mitochondria (Millar et al., 2001; Heazlewood et al., 2004). However, it can presently not be excluded that one or both of these proteins can additionally be present in another subcellular compartment, since PMH2 (At3g22330) has recently been identified in a proteome analysis of nucleoli, but this localization has not been further analyzed (Pendle et al., 2005).

In the mitochondrial lysate several spots are detected after IEF and SDS PAGE. Presently we can only speculate about the appearance of different protein spots, which were observed in all experiments performed. These might be experimental artifacts such as protein carbamylation. or oxidation . even though the isoelectric focusing conditions were chosen to avoid such effects. In addition the differential presence of the PMH proteins in these spots suggests that they represent PMH proteins with differing posttranslational modifications. However, further experiments are required to clarify this issue.

Distinct expression patterns suggest different roles of PMH1 and 2

PMH1 and 2 share 77 % identical amino acids and thus are very similar to each other. In addition each protein contains a characteristic C-terminus, which is in both proteins predominantly composed of serine and glycine. These similarities suggest similar functions of both proteins, however, there are clear differences in terms of spatiotemporal transcription patterns of each gene. Also the response of PMH1 expression to cold is stronger than the induction of PMH2 and only the PMH2 promoter activity is induced by wounding. From these experiments it can be concluded that despite the high similarity of the two proteins, the polypeptides might fulfill different roles in plant mitochondria. PMH1 is expressed only in certain situations and might support PMH2 in its function. For instance during flowering, which is an energy consuming process, elevated mitochondrial activity is required. Likewise

cold stress might be a situation in which PMH2 activity is needed and in which it is supported by PMH1, which is stronger induced under this environmental condition than PMH2. Although the genevestigator expression analysis tool (https://www.genevestigator.ethz.ch/) cannot discriminate between PMH1 and 2 genes (both genes are represented by a single oligonucleotide on the ATH1 gene chip) the cold induction is confirmed by a meta-profile analysis. This investigation also reveals enhanced expression after heat stress, in seeds particularly 3 h after imbibition and in stems, conditions and tissues not inspected in our expression studies. In contrast to our northern and histochemical GUS staining analyses, the strong expression in flowers or parts of them is not indicated by the meta-profile analysis. Likewise no response to wounding is found by this gene expression analysis, although enhanced *PMH* mRNA levels are found upon biotic stress caused by different pathogens. However, nothing is known about a function of a DEAD-box protein in response to wounding or pathogen attack.

PMH1 and 2 contain serine- and glycine-rich C-termini

Both PMH proteins contain approximately 100 amino acids long C-termini rich in Arg, Gly and Ser. Such termini, although in different lengths, are widespread among RNA binding proteins (Lorkovic and Barta, 2002; Vermel et al., 2002), and in *A. thaliana* there is a number of putative RNA helicases, which also contain such particular C-termini (Aubourg et al., 1999). One of these proteins, At5g26743, shares 62 % identical amino acids with the core regions of PMH1 and 2 and has a 30 amino acid long Ser/Gly-rich stretch within the C-terminus. This protein is predicted to be targeted to chloroplasts, where its homologue in spinach has indeed very recently been detected in a proteomic approach investigating plastid proteins (Baginsky et al., 2007). This protein might thus be the plastid ortholog of the two mitochondrial PMH proteins. An approximately 50 amino acids Gly/Arg-rich C-terminus is also present in the nuclear DEAD-box protein PRH75 (At5g62190). The recombinant

extension of this protein was found to bind to RNA and is therefore considered to be an independent RNA binding module (Lorkovic et al., 1997). The same function can be assumed for the C-termini of PMH1 and 2 suggesting a direct interaction of these proteins with RNA, which is consistent with the loss of the association of the PMH proteins from the complexes upon RNase treatment. In *A. thaliana* at least the mitochondrial RNA binding protein RBP1a containing a 30 amino acid long glycine-rich C-terminus is also inducible by cold (Vermel et al., 2002) and it would be interesting to see whether this protein is present in one of the PMH1/2 containing complexes.

PMH1 and/or PMH2 are associated with large RNA-containing complexes

As indicated by two-dimensional BN/SDS-PAGE and discontinuous sucrose gradient centrifugation both or at least one of the PMH proteins are/is part of large protein complexes. The exact composition of these complexes is presently unclear but extrapolating from other DEAD-box protein containing complexes it can be assumed that several different proteins are present in these complexes. Certainly RNA is a component of the complexes as clearly indicated by the observation that RNase digestion disrupts the complexes themselves or at least prevents the interaction of the PMH proteins with the complexes. In the latter case the DEAD-box proteins would directly interact with the RNA, which would be possible through the glycine-rich C-termini as mentioned above, while the former would indicate a central role of RNA in the architecture of the complex. Recently another RNA-dependent protein complex has been observed in plant mitochondria, which contains a FLAG-tagged version of the PPR protein RF592, the restorer of the *pcf*-associated cytoplasmic male sterility in petunia (Gillman et al., 2007). The other components of this complex are still unknown.

The dependence of complex formation on RNA might also explain the size variability of the complex, which ranges from the monomeric form up to 1,500 kDa as estimated from BN/SDS PAGE. Within this size range distinct intermediate complexes are reproducibly observed. In

addition the size variability is also indicated by the presence of the PMH proteins in different fractions of the sucrose gradients, suggesting the size variation not to be the result of a particular preparative procedure. This distribution is highly reproducible and independent from the presence of aminocaproate which can destabilize protein complexes (data not shown) (Novakova et al., 2006).

Several explanations are possible for this size variation: First, the different complexes could represent different degradation intermediates, but the high reproducibility and the influences of $MgCl_2$ and cold do not support this explanation. Second, the pattern might be attributed to assembly intermediates, but such intermediates are typically rather low in abundance and should not be detectable at such high levels. Third, this pattern might indicate the presence of mature complexes with varying associated components, different RNA molecules, different proteins or both. This has for instance been observed for p53. This multifunctional protein is present in different complexes as indicated by a western blot analysis of whole cell extracts also separated by two dimensional BN/SDS PAGE (Camacho-Carvajal et al., 2004). Here also protein spots and a smear are observed in a horizontal line indicative of a protein being present in several distinct complexes.

Searching for a function of the PMH1 and 2 DEAD-box proteins

The function(s) of PMH1 and 2 remain(s) unclear. The distribution of these proteins and of the rRNAs in the discontinuous sucrose gradients is too different to reflect a stable association with mature ribosomes, although a weak interaction of PMH1 and 2 is possible (Fig. 6). Thus a function of these proteins in translation initiation cannot be excluded. To obtain more information about the functional role of these proteins we established the respective knockout mutants. However, a preliminary characterization of these plants grown under normal conditions as well as under different temperatures did not reveal any obvious phenotype (data

not shown). Thus further detailed studies are required to uncover the physiological function of these PMH proteins.

Maybe the PMH proteins are involved in RNA secondary structure rearrangements which are required for the maintenance of cellular functions at reduced and elevated temperatures, as has been suggested for prokaryotic DEAD-box RNA helicases (Owttrim, 2006). Such a scenario could also be relevant for plant mitochondria, which are exposed to highly differing environmental temperatures. The studies reported here will form a convenient platform for further detailed investigations to elucidate the exact function of the mitochondrial DEAD-box proteins PMH1 and 2.

MATERIALS AND METHODS

Subcellular localization studies

DNA fragments representing the N-terminal parts of PMH1 and PMH2, respectively, were amplified with the following primer pairs pmh1GFP1.5 (5'-CACTCTCTGGGATCCGAAAATG)/pmh1GFP1.3 (5'-GCGGATCCTAACATGAAAGTCTCTCAC), pmh1GFP1.5/pmh1GFP2.3 (5'-CGAATCTCGGATCCCTCCATCG), pmh2GFP1.5 (5'-CACCTGGATCCGAAAATGATCAC)/pmh2GFP1.3 (5'-ATGGATCCCTTTCGCTTCAACACC) and pmh2GFP1.5/pmh2GFP2.3 (5'-GCGGATCCCAAGCCCATCACCACCAAC). PCRs were performed using oligo(dT)-primed cDNA from *A. thaliana* total RNA as template and BD Advantage 2 polymerase following the instructions given by the manual (Takara, Europe). The cDNA products, which code for 69 and 100 amino acids of the PMH1 protein and 62 and 104 amino acids of the PMH2 polypeptide, respectively, were digested with BamHI and ligated into the corresponding site in the psmGFP4 vector (Davis and Vierstra, 1998). After sequence analysis

correct clones were transformed into tobacco protoplasts. Protoplast isolation, transformation, staining and inspection by fluorescence microscopy were done as described previously (Koop et al., 1996; Däschner et al., 2001).

Northern hybridization

Arabidopsis thaliana ecotype Col-0 was grown on standard soil supplemented with Osmocote® Exact Mini (Scotts, Geldermalsen, Netherlands) or on Murashige Skoog (MS) medium in a growth chamber under a 16 h (160-200 µmol/m^2s, 23°C)/ 8h light dark (21°C) regime. Tissues from adult plants were harvested from three to four week-old plants. Seedlings were grown for seven days and then transferred to cold or kept at 23°C as control. RNA was isolated using an RNeasy Plant Mini kit following the manual given by the manufacturer (Qiagen, Hilden, Germany). For northern analysis about 10 µg of total RNA were denatured with glyoxale, separated on agarose gels, transferred to Hybond N nylon membranes and hybridized according to standard protocols or to a protocol provided by the manufacturer (GE Healthcare). Probes used for hybridizations correspond to the long cDNA fragments used for studying subcellular targeting. Loading of the gels was monitored by hybridization with oligonucleotide P18SrRNA (5'-AAGCATATGACTACTGGCAGG) complementary to nuclear/cytoplasmic 18S rRNA sequences.

Histochemical GUS staining

To study *in vivo* promoter activities potential promoter regions corresponding to sequences from -1981 to +14 (PMH1) and -2067 to +3 (PMH2) with respect to the ATG (+1) were amplified with primer pairs pmh1prom5' (5'-ATAGTCGACTCAGAAACTCTAGAATCC)/pmh1prom3' (5'-ATAGTCGACTGTGCTAATCATTTTCAG) and pmh2prom5'Xba2 (5'-ACTCTAGAATGGTAGCCATCTCAACACC)/pmh1prom3'Xba (5'-

TATCTAGACATTTTCAGATTCAGGTGTTC) on total DNA extracted with a DNeasy Plant Mini kit as outlined by the manufacturer (Qiagen). After amplification PCR fragments were cloned upstream of the GUS genes into vector pBecks19/101, transformed into *A. thaliana* plants by floral dip and selected on kanamycin containing MS medium (Clough and Bent, 1998). Histochemical staining was done with plants derived from five independent transformation events per gene as given in a previously described protocol (Hull and Devic, 1995).

Blue native/urea SDS PAGE

Mitochondria were isolated and purified from an *A. thaliana* Col-0 cell suspension culture as described before (Klein et al., 1998). To investigate the response to cold treatment, the cell cultures were first grown at normal growth conditions and then kept at 4°C for 18 h prior to isolation of mitochondria. Two-dimensional blue native/sodium dodecyl sulfate polyacrylamide gel electrophoresis (BN/SDS PAGE) was done according to a protocol established for plant mitochondria with the following specific parameters (Eubel et al., 2005). About 1 mg mitochondrial protein (= 10 mg mitochondria) was resuspended in 80 µl solubilization buffer (750 mM aminocaproate, 50 mM BisTris pH 7.0, 0.5 mM EDTA). Proteins were solubilized by adding 15 µl dodecylmaltosid (DDM, 10 % w/v) to a final concentration of 1.3 % (w/v). Optionally 10 mM $MgCl_2$ or 1 µg RNaseA/100 µl protein lysate were added. The solution was kept on ice for 10 min and samples were then centrifuged at 18,000 x g for 1 h to remove insoluble constituents. After adding 20 µl of Coomassie loading buffer (10 % [w/v] Coomassie in 750 mM aminocaproate) to the supernatant, the sample was loaded onto 3 % to 16 % (w/v) acrylamide gradient gels for separation in the first dimension. Separation in the second dimension was carried out with urea gels (12.5 %). Gels were then either stained with Coomassie colloidal blue or proteins were blotted onto PVDF membranes. Immunodetection analysis was carried out with a polyclonal serum against peptides NH_2-

AQRERTLAGFRDGNF and NH_2-ELPSIAVERGSASMFE corresponding to amino acids 397-411 and 485-500, respectively, relative to the ATG of PMH1. Peptide synthesis, immunization of rabbits as well as recovery of the sera was done by EUROGENTEC (Liege, Belgium). All procedures of immunodetection followed standard protocols (Sambrook and Russel, 2001).

Isoelectric focusing

Isoelectric focusing (IEF) was performed with the IPGphor System following the manufacturer's instructions (GE Healthcare). About 20 mg of mitochondria were resolved in 200 µl lysis buffer (2 M thiourea, 5 M urea, 2 % CHAPS, 2 % SB 3-10, 40 mM Tris Base, 2 mM TBP, 0,2 mM PMSF) for 1 h and insoluble constituents were removed by centrifugation at 18,400 x g for 20 min. Prior to separation by IEF proteins were precipitated by adding 3 volumes of precipitation solution (90 % (v/v) acetone, 10 % (v/v) methanol, 10 mM DTT), incubation over night and centrifugation for 15 min at 20,000 x g. Proteins were resuspended in rehydration solution (2 M thiourea, 5 M urea, 1 % (w/v) CHAPS, 1 % (w/v) SB 3-10, a trace of bromophenol blue, 2 mM TBP, 0.5 % IPG buffer). Separation was done on immobiline nonlinear DryStrip gels pH 3 to 11 and 7 to 11, respectively, under the following conditions: 50 µA per strip at 21°C. For pH 3-11 gradients: rehydration 12 h, step 1: step and hold, 200 V, 3h; step 2: step and hold, 500 V, 3 h; step 3: gradient 1,000 V, 3h, step 4: gradient, 8,000 V, 6 h; step 5: step and hold, 8,000 V, 7 h. pH gradient 7-11: rehydration 24 h, step 1: step and hold, 200 V, 2 h, step 2: step and hold, 500 V, 2 h, step 3: gradient, 4,000 V, 4 h, step 4: gradient, 8000 V, 5 h, step 5: step and hold, 8,000 V 7 h. After focusing gel strips were equilibrated in buffer A (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, a trace of bromophenol blue, 1 % (w/v) DTT) or buffer B, which is identical to buffer A except that DTT is replaced by 2. 5 % IAA. Subsequently Tris–Tricine PAGE was carried out according to a previously described protocol (Schagger and von Jagow, 1987) and

proteins were visualized with colloidal Coomassie, investigated by immunodetection analysis or examined by mass spectrometry (MS).

For MS, visible protein spots were excised from the gel and digested with trypsin as described previously (Shevchenko et al., 1996). Peptides were extracted, separated and analyzed (i.e. sequenced) by nanoLC-coupled ESI MSMS using a hybrid triple quadrupole/linear iontrap mass spectrometer (4000 Qtrap, Applied Biosystems) under standard conditions. For protein identification, fragment spectra were searched against the NCBInr database using Mascot as search engine.

Fractionation on sucrose gradients

For sub fractionation of mitochondrial protein complexes 100 mg purified organelles were solved in solubilization buffer (see above) in the presence of 200 mM Tris-HCl pH 8.0, 35 mM $MgCl_2$, 25mM EGTA, 200 mM sucrose, 40 mM EDTA, 2 % (w/v) DDM, 0.5 mg/ml Heparin, 10 mM ß-mercaptoethanol, 500 mM chloramphenicol and optional 300 mM KCl (Raczynska et al., 2006). The supernatant containing the mitochondrial proteins was then loaded onto discontinuous sucrose gradients, with sucrose concentrations ranging in 5 % steps from 15 to 55 % in gradient buffer containing 40 mM Tris-HCl pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA; 0.5 mg/ml heparin; 10 mM ß-mercaptoethanol and optional 100µg/ml chloramphenicol. Gradients were centrifuged at 217,000 x g for 4 h at 4°C. Fractions of 1 ml were taken from top to bottom each corresponding to a complete step. Proteins (50 µl) from each of the fractions were directly analyzed by immunodetection analysis, while RNA was precipitated from 800 µl of each fraction by the addition of 1 volume of 8 M guanidine HCl and 4 volumes of 100 % EtOH and an incubation -20°C over night. A second precipitation was done by addition of 0.1 volume 3 M sodium acetate ph 5.2 and 2 volumes of 100 % EtOH. RNA was inspected by agarose gel electrophoresis and ethidium bromide staining.

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LITERATURE CITED

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796-815

Aubourg S, Kreis M, Lecharny A (1999) The DEAD box RNA helicase family in Arabidopsis thaliana. Nucleic Acids Res **27:** 628-636

Baginsky S, Grossmann J, Gruissem W (2007) Proteome analysis of chloroplast mRNA processing and degradation. J Proteome Res **6:** 809-820

Bentolila S, Alfonso AA, Hanson MR (2002) A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Proc Natl Acad Sci U S A **99:** 10887-10892

Binder S, Brennicke A (2003) Gene expression in plant mitochondria: transcriptional and posttranscriptional control. Phil Trans R Soc Lond B **358:** 181-199

Boudet N, Aubourg S, Toffano-Nioche C, Kreis M, Lecharny A (2001) Evolution of intron/exon structure of DEAD helicase family genes in *Arabidopsis*, *Caenorhabditis*, and *Drosophila*. Genome Res **11:** 2101-2114

Brown GG, Formanova N, Jin H, Wargachuk R, Dendy C, Patil P, Laforest M, Zhang J, Cheung WY, Landry BS (2003) The radish Rfo restorer gene of Ogura cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. Plant J **35:** 262-272

Camacho-Carvajal MM, Wollscheid B, Aebersold R, Steimle V, Schamel WW (2004) Two-dimensional Blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates: a proteomics approach. Mol Cell Proteomics **3:** 176-182

Clough SJ, Bent AF (1998) Floral dip: asimplified method for Agrobacterium-mediated transfromation of Arabidopsis thaliana. Plant J **16:** 735-742

Däschner K, Couee I, Binder S (2001) The mitochondrial isovaleryl-coenzyme a dehydrogenase of *Arabidopsis* oxidizes intermediates of leucine and valine catabolism. Plant Physiol **126:** 601-612

Davis SJ, Vierstra RD (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol **36:** 521-528

Desloire S, Gherbi H, Laloui W, Marhadour S, Clouet V, Cattolico L, Falentin C, Giancola S, Renard M, Budar F, Small I, Caboche M, Delourme R, Bendahmane A (2003) Identification of the fertility restoration locus, Rfo, in radish, as a member of the pentatricopeptide-repeat protein family. EMBO Rep **4:** 588-594

Eubel H, Braun HP, Millar AH (2005) Blue-native PAGE in plants: a tool in analysis of protein-protein interactions. Plant Methods **1:** 11

Eubel H, Jansch L, Braun HP (2003) New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of complex II. Plant Physiol **133:** 274-286

Gagliardi D, Binder S (2007) Expression of the plant mitochondrial genome. *In* D Logan, ed, Plant mitochondria. Blackwell Publishing, Ames, IA, USA, pp pp. 50-95

Gillman JD, Bentolila S, Hanson MR (2007) The petunia restorer of fertility protein is part of a large mitochondrial complex that interacts with transcripts of the CMS-associated locus. Plant J **49:** 217-227

Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Millar AH (2004) Experimental analysis of the *Arabidopsis* mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell **16:** 241-256

Hoffmann M, Binder S (2002) Functional importance of nucleotide identities within the pea *atp9* mitochondrial promoter sequence. J Mol Biol **320:** 943-950

Hull GA, Devic M (1995) The beta-glucuronidase (gus) reporter gene system. Gene fusions, spectrophotometric, fluorometric, and histochemical detection. *In* H Jones, ed, Methods Mol Biol, Vol 49. Humana Press Inc., Totowa, NJ, pp 125-141

Kazama T, Toriyama K (2003) A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp*6 RNA of cytoplasmic male-sterile rice. FEBS Let **544:** 99-102

Klein M, Binder S, Brennicke A (1998) Purification of mitochondria from Arabidopsis. Methods Mol Biol **82:** 49-53

Koizuka N, Imai R, Fujimoto H, Hayakawa T, Kimura Y, Kohno-Murase J, Sakai T, Kawasaki S, Imamura J (2003) Genetic characterization of a pentatricopeptide repeat protein gene, orf687, that restores fertility in the cytoplasmic male-sterile Kosena radish. Plant J **34:** 407-415

Koop HU, Steinmuller K, Wagner H, Rossler C, Eibl C, Sacher L (1996) Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. Planta **199:** 193-201

Kruft V, Eubel H, Jansch L, Werhahn W, Braun HP (2001) Proteomic approach to identify novel mitochondrial proteins in *Arabidopsis*. Plant Physiol **127:** 1694-1710

Linder P (2006) Dead-box proteins: a family affair--active and passive players in RNP-remodeling. Nucleic Acids Res **34:** 4168-4180

Lorkovic ZJ, Barta A (2002) Genome analysis: RNA recognition motif (RRM) and K homology (KH) domain RNA-binding proteins from the flowering plant Arabidopsis thaliana. Nucleic Acids Res **30:** 623-635

Lorkovic ZJ, Herrmann RG, Oelmuller R (1997) PRH75, a new nucleus-localized member of the DEAD-box protein family from higher plants. Mol Cell Biol **17:** 2257-2265

Marchfelder A, Binder S (2004) Plastid and plant mitochondrial RNA processing and RNA stability. *In* H Daniell, C Chase, eds, Molecular Biology and Biotechnolgy of Plant Organelles. Springer, Dordrecht, Netherlands, pp 261-294

Millar AH, Sweetlove LJ, Giegé P, Leaver CJ (2001) Analysis of the *Arabidopsis* mitochondrial proteome. Plant Physiol **127:** 1711-1727

Mingam A, Toffano-Nioche C, Brunaud V, Boudet N, Kreis M, Lecharny A (2004) DEAD-box RNA helicases in *Arabidopsis thaliana*: establishing a link between quantitative expression, gene structure and evolution of a family of genes. Plant Biotechnol J **2:** 401-415

Novakova Z, Man P, Novak P, Hozak P, Hodny Z (2006) Separation of nuclear protein complexes by blue native polyacrylamide gel electrophoresis. Electrophoresis **27:** 1277-1287

Owttrim GW (2006) RNA helicases and abiotic stress. Nucleic Acids Res **34:** 3220-3230

Pendle AF, Clark GP, Boon R, Lewandowska D, Lam YW, Andersen J, Mann M, Lamond AI, Brown JW, Shaw PJ (2005) Proteomic analysis of the *Arabidopsis* nucleolus suggests novel nucleolar functions. Mol Biol Cell **16:** 260-269

Perrin R, Lange H, Grienenberger JM, Gagliardi D (2004a) AtmtPNPase is required for multiple aspects of the 18S rRNA metabolism in *Arabidopsis thaliana* mitochondria. Nucleic Acids Res **32:** 5174-5482

Perrin R, Meyer EH, Zaepfel M, Kim YJ, Mache R, Grienenberger JM, Gualberto JM, Gagliardi D (2004b) Two exoribonucleases act sequentially to process mature 3'-ends of atp9 mRNAs in *Arabidopsis* mitochondria. J Biol Chem **279:** 25440-25446

Raczynska KD, Le Ret M, Rurek M, Bonnard G, Augustyniak H, Gualberto JM (2006) Plant mitochondrial genes can be expressed from mRNAs lacking stop codons. FEBS Let **580:** 5641-5646

Rocak S, Linder P (2004) DEAD-box proteins: the driving forces behind RNA metabolism. Nat Rev Mol Cell Biol **5:** 232-241

Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. *In*, Ed Ed 3. Cold Spring Habror Laboratory Press, Cold Spring Harbor, NY

Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **166:** 368-379

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem **68:** 850-858

Vermel M, Guermann B, Delage L, Grienenberger JM, Marechal-Drouard L, Gualberto JM (2002) A family of RRM-type RNA-binding proteins specific to plant mitochondria. Proc Natl Acad Sci U S A **99:** 5866-5871

Wang Z, Zou Y, Li X, Zhang Q, Chen L, Wu H, Su D, Chen Y, Guo J, Luo D, Long Y, Zhong Y, Liu YG (2006) Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. Plant Cell **18:** 676-687

FIGURE LEGENDS

Figure 1. PMH1 and PMH2 are located in mitochondria. Images of tobacco protoplasts expressing the PMH1:GFP (69 amino acid construct) and the PMH2:GFP (104 amino acid construct) fusion proteins, respectively, were taken with filters allowing the detection of the GFP fluorescence and the chlorophyll autofluorescence (left) and a filter optimized for visualization of MitoTracker fluorescence (right). Space bars correspond to ten µm.

Figure 2. Transcription of *PMH1* and *PMH2*. Northern blot analysis shows that *PMH1* transcripts are predominantly present in flowers (f), while *PMH2* mRNAs are almost equally present in roots (r), seedlings (s) and leaves (l) and are approximately three-fold more

abundant in flowers (compared to seedling, upper part). Steady state transcript levels of both genes are elevated in cold treated seedlings (4°C for 24 h, s4°) in comparison to control plants cultivated at 23°C (s23°). The quality of the RNA and the loading of the gel were checked by hybridization with an 18S rRNA–specific oligonucleotide probe (lower parts).

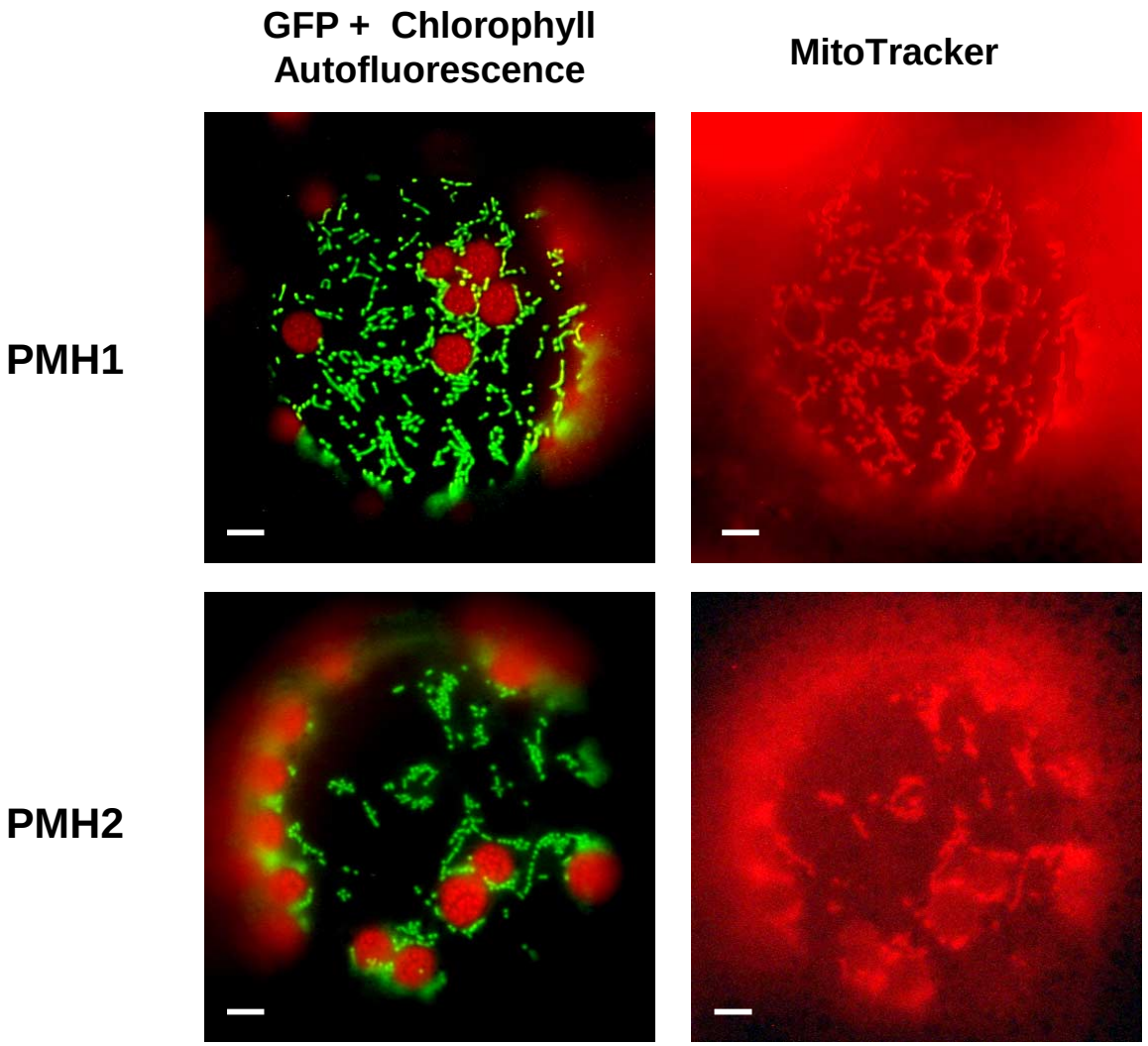
Figure 3. Histochemical analysis of transgenic *A. thaliana* plants expressing the GUS reporter gene under the *PMH1* and *PMH2* promoters. Six, nine and 16 day-old seedlings grown on MS medium (upper part) as well as different organs of adult plants cultivated on soil (lower part, from left to right: basis of leaves, roots, flowers and siliques) were analyzed. Further details are given in the text.

Figure 4. The two DEAD-box proteins are detectable in mitochondria from cell suspension cultures. Total mitochondrial protein is analyzed by isoelectric focusing (IEF, first dimension) and Tricine-SDS PAGE (second dimension). (A) Coomassie staining revealed spots in the area, where PMH2 is expected from previous studies (pH 10/60 kDa, dashed box). (B) An immunodetection analysis with the PMH1/2 antiserum detects specific spots in this area. (C and D) Enhanced resolution of the proteins was achieved on pH 7 to 11 gradients. A section of the stained gel (C) shows spots in expected size and pH range (indicated by arrows a and b). Relevant spots (indicated by open rectangles) were excised from the gel and analyzed by mass spectrometry. In all spots of the upper row PMH1 and/or 2 are the predominant proteins. (D) An immunodetection analysis indicates the presence of the PMH proteins in the upper row of the proteins seen between pH 9 to 10.

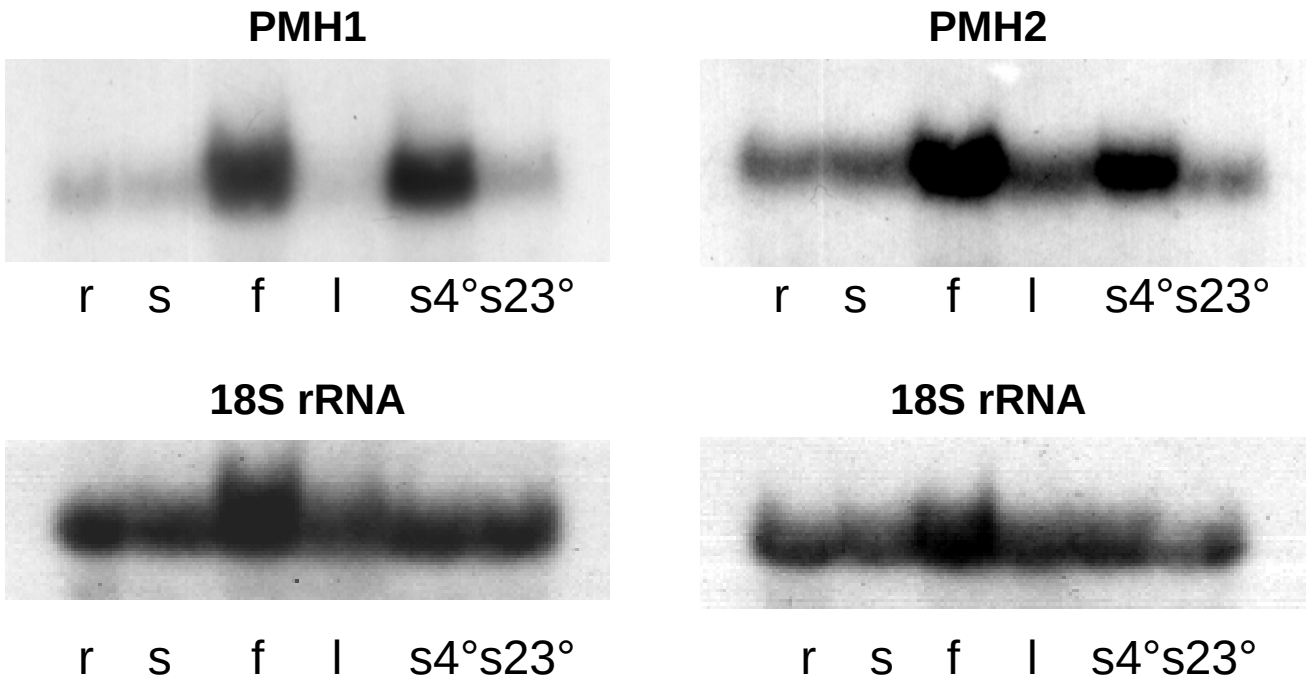
Figure 5. PMH1/2 are bound in RNA-dependent high molecular weight complexes. DDM-solubilized total mitochondrial protein from cell suspension culture was separated in a first dimension by blue native PAGE followed by a urea SDS PAGE in the second dimension (A)

Coomassie stained gel, (B) immunodetection analysis of DDM-solubilized protein with @PMH1/2 antibodies, (C) as (B) after separation of RNase A treated mitochondrial lysates, (D) immunodetection analysis with @PMH1/2 after cold treatment of the suspension culture for 18 h and (E) solubilization in the presence of 10 mM $MgCl_2$. Sizes in the first dimension are estimated from complexes $I+III_2$ (1,500 kDa), I (1,000 kDa), V (580 kDa), III_2 (480), formate dehydrogenase (200 kDa) and aconitase (100 kDa) (Eubel et al., 2005).

Figure 6. Fractionation of mitochondrial ribonucleoprotein complexes. Total mitochondrial protein solubilized in the absence (A) and presence of 300 mM KCl (B) was fractionated on discontinuous sucrose gradients. Fraction 1 corresponds to the sample buffer loaded onto the gradient. Fraction 2 corresponds to 15 % sucrose, 3 to 20 %, 4 to 25%, 5 to 30 %, 6 to 35 %, 7 to 40 %, 8 to 45 %, 9 to 50 % and 10 to 55 % sucrose, respectively. Proteins of fractions 1 to 9 were analyzed by immunodetection with the @PMH1/2 antibody. RNA of fractions 1 to 10 was inspected by agarose gel electrophoresis and ethidium bromide staining (RNA). Fractionation was done in a buffer optimized for the separation of intact ribosomes and polysomes.. Positions of 26S rRNA (26S) and 18S rRNA (18S) are indicated.



Matthes et al. Fig. 1



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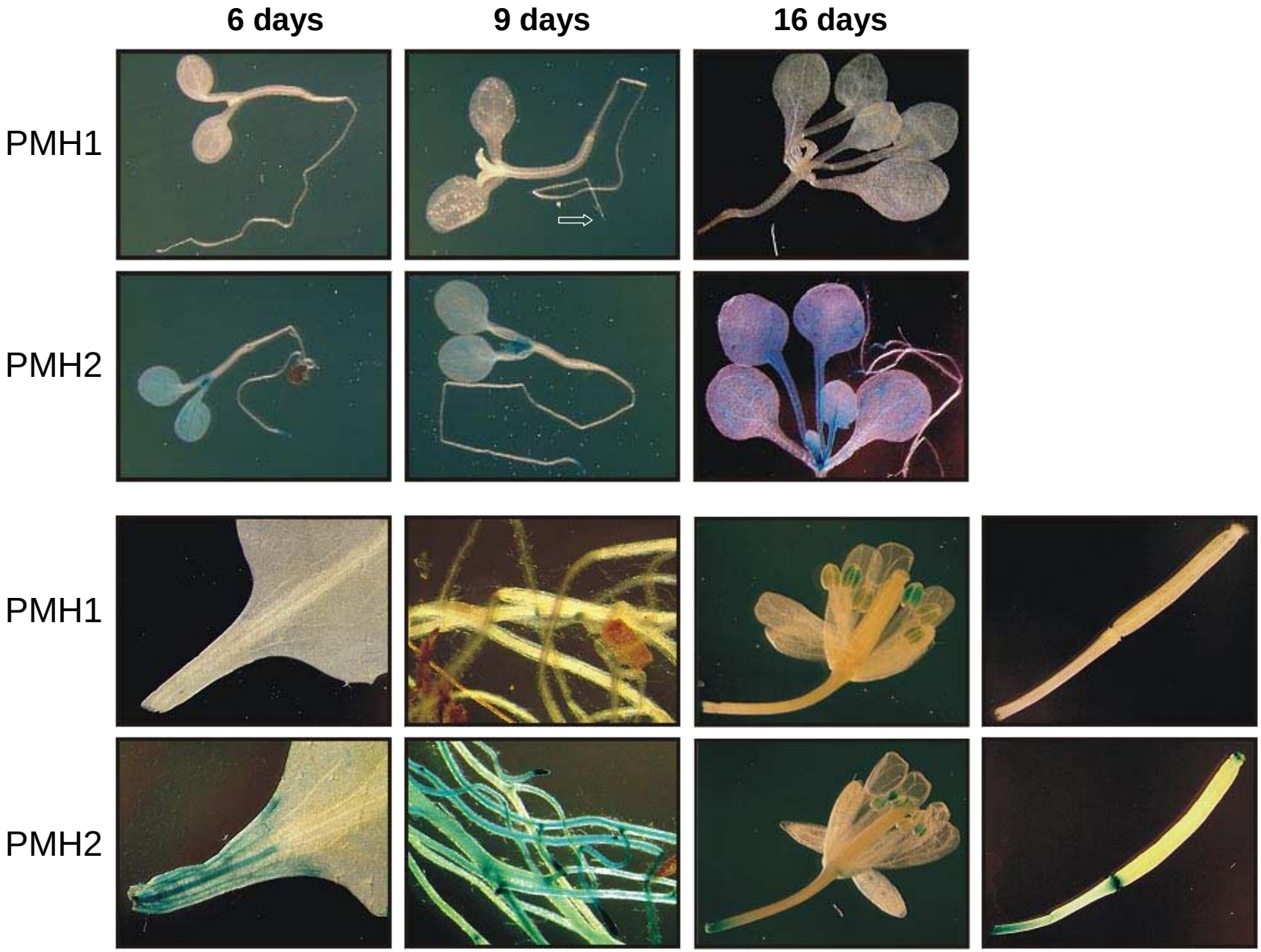

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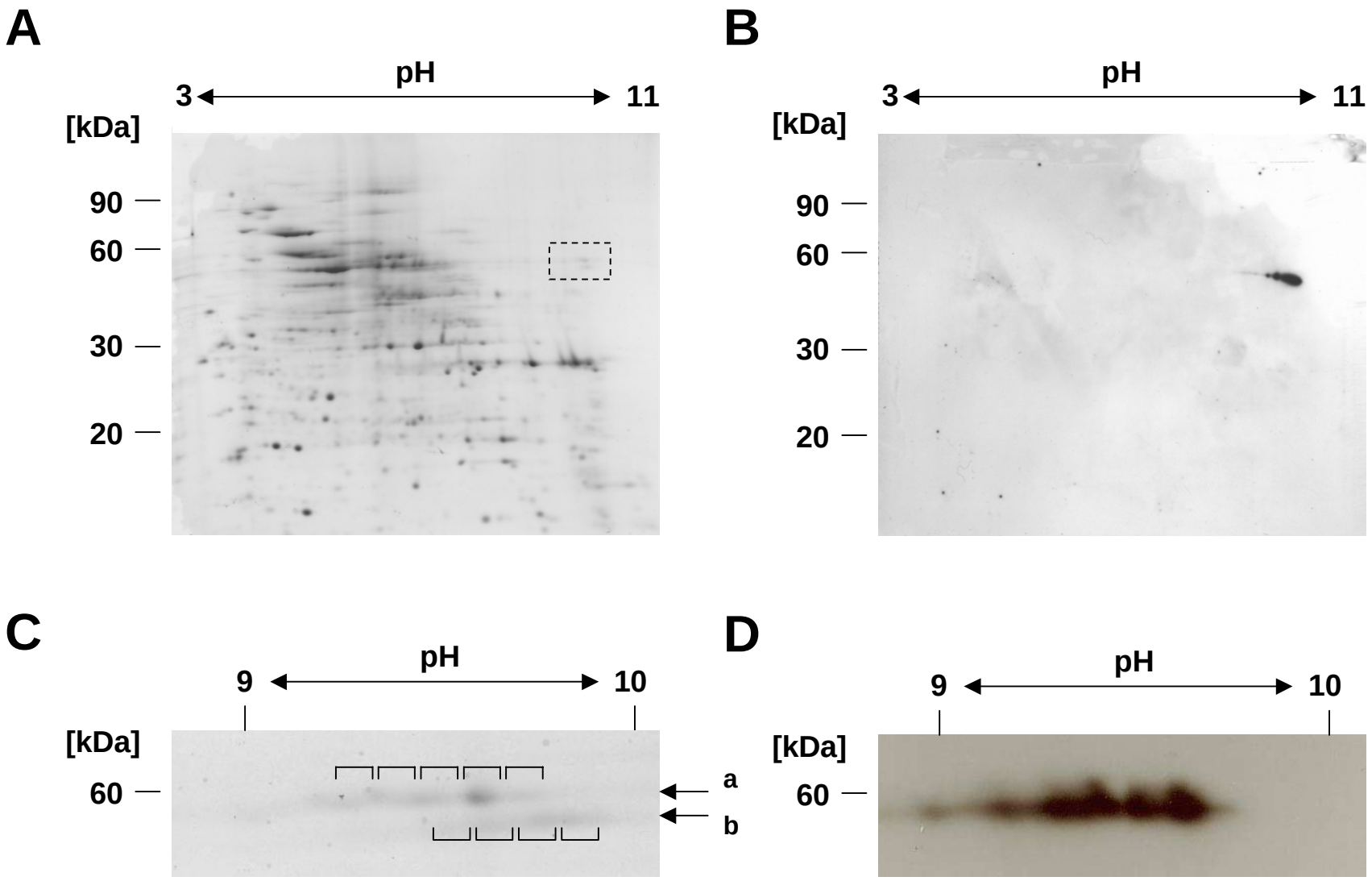

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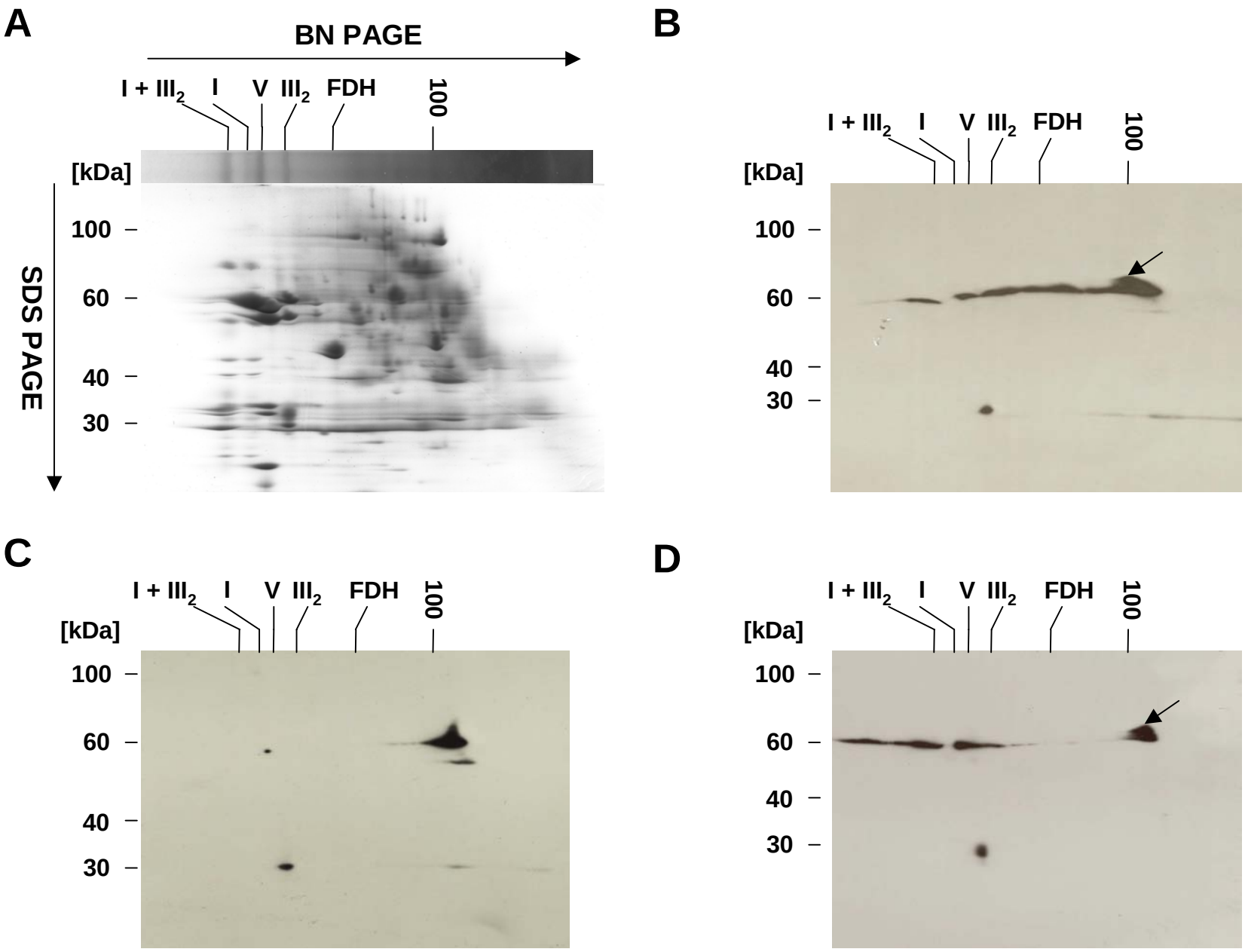
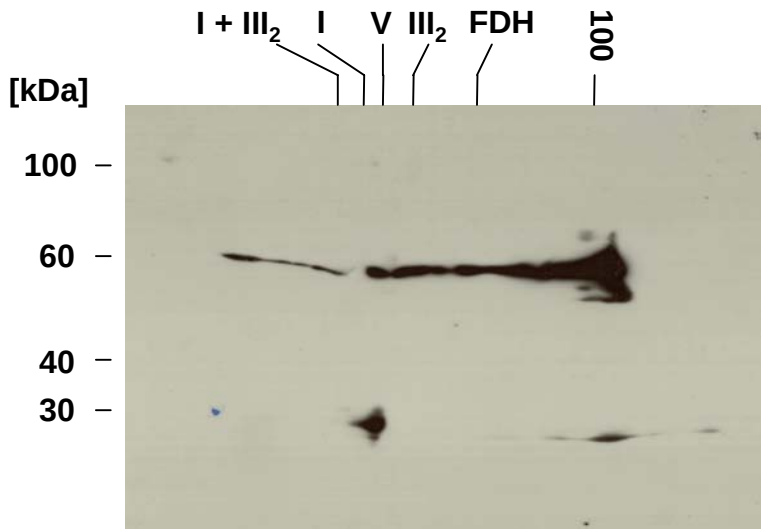


Matthes et al. Fig. 5A - D

E



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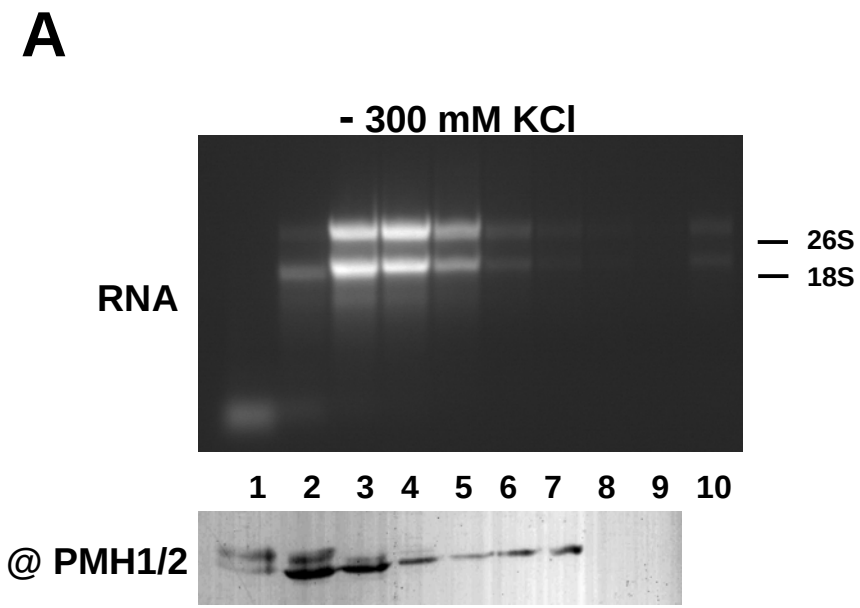

Matthes et al Fig. 6