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Arabidopsis thaliana poly (A) binding protein 2 (PAB2) functions in yeast translational and mRNA decay processes

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Summary

The single yeast gene (*PAB1*) encoding poly (A) binding protein (PABP) has several roles in post-transcriptional processes, including translation initiation and mRNA decay. PABP is encoded by a large gene family in plants. Within *Arabidopsis thaliana*, the several characterized PABP genes exhibit an extreme degree of sequence divergence and are differentially expressed. *Arabidopsis* PAB2 is expressed in distinct tissues or during defined developmental windows in most plant organs. In this study we demonstrate that *PAB2* restores viability to a yeast *pab1* mutant strain. Yeast strains containing wild-type, null (PAB2^{s}) and temperature sensitive (PAB2^{ts}) alleles of *PAB2* were used to explore the molecular functions of the plant protein. PAB2 can participate in poly (A) tail shortening, thus demonstrating that it interacts with the yeast poly(A) nuclease complex. *PAB2* is required for translation, helping to maintain intact polysome structures. Consistent with its role in translation initiation, poly (A) was found to enhance PAB2 binding to *Arabidopsis* eIF-iso4G *in vitro*. In addition, PAB2 can partially restore the linkage between deadenylation, decapping and mRNA decay in yeast. Taken together, our results suggest that *Arabidopsis* PAB2 participates in many of the same complex post-transcriptional processes identified for yeast PAB1, and is functionally distinct from other characterized *Arabidopsis* PABPs.

Introduction

Poly (A) binding protein (PABP) is the major protein complexed with poly (A) tails of eukaryotic mRNAs. PABP genes have been cloned from a variety of organisms ranging from yeast to humans. Sequence analysis reveals that all PABP genes have a similar primary structure, comprising of four RNA recognition motifs (RRM) in the amino terminal two-thirds of the protein and a structurally undefined, albeit conserved, domain in the carboxyl terminal region of the protein. Interestingly, the individual RRMs in a PABP are more closely related to their counterparts in other PABPs than to other RRMs in the same protein. This sequence divergence suggests that the individual RRMs may have ancient and distinct molecular functions. Consistent with this hypothesis, *in vitro* studies using yeast, *Xenopus* and human PABPs have found that individual RRMs differ in their RNA binding affinities and specificities: RRM 1 and 2 have the greatest specificity (compared to RRM3 and RRM4) for binding poly (A) whereas RRM3 and RRM4 may be involved in binding polypyrimidine-rich RNA and/or other RNA sequences (Burd *et al.*, 1991; Deardorff and Sachs, 1997; Kuhn and Pieler, 1996). The carboxy-terminal half of the protein has been implicated in homodimerization activity on PABP and, through its interaction with RRM3 and 4, in organizing PABP at a regular spacing on a poly (A) substrate (Kuhn and Pieler, 1996).

PABP is encoded by a single, essential gene in yeast (Sachs *et al.*, 1987). *In vivo* evidence for PABP involvement in translation was first obtained using conditional lethal mutants of yeast *PAB1* (Sachs and Davis, 1989). Subsequently, it was demonstrated that yeast PAB1 recruits the 43S pre-initiation complex to the mRNA through its physical interaction with initiation factor eIF4G of the 5′ cap-binding complex in a poly (A)-dependent manner (reviewed in Sachs *et al.*, 1997). There is probably also similar functional interaction between the 5′ and 3′ ends of mRNA in higher eukaryotes, as the interaction between PABP and eIF4G was recently demonstrated in wheat and humans (Craig *et al.*, 1998; Imataka *et al.*, 1998; Le *et al.*, 1997b; Piron *et al.*, 1998).

A role for PABP in poly (A) tail length control was proposed based on findings that depletion or inactivation

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of yeast PAB1 leads to accumulation of mRNAs with abnormally long poly (A) tail lengths similar to the length found in pre mRNAs in the nucleus (Sachs and Davis, 1989). Subsequently, a PAB1-dependent poly (A) nuclease (PAN) complex in yeast was purified and the genes encoding some of the subunits were cloned (Boeck *et al*., 1996; Brown *et al*., 1996). Further investigation using yeast *pan* mutants found that the PAB1-dependent PAN activity is required by newly synthesized mRNAs to achieve message-specific maximal poly (A) tail length. These findings have thus implicated yeast PAB1 in a new post-transcriptional regulatory step (Brown and Sachs, 1998). Of possible relevance is the recent finding which also implicates PABP in nuclear processes (Afonina *et al*., 1998; Amrani *et al*., 1997; Mangus *et al*., 1998).

Another role for PABP is influencing the stability of mRNAs. Using a polysome-based mammalian *in vitro* degradation system, it was shown that PABP conferred stability on mRNAs by binding to their poly (A) tails and preventing them from degrading (Bernstein *et al*., 1989). In mammalian cells, PABP has been shown *in vitro* to be essential for the stability of α-globin mRNA through its interaction with the α-globin mRNA stability complex, called α-complex (Wang *et al*., 1999). Furthermore, the ability of yeast PAB1 to stabilize mRNAs has been demonstrated (Coller *et al*., 1998). In yeast, the major degradation pathway of mRNAs initiates with the shortening of poly (A) tails, followed by decapping and subsequent degradation of the body of the message (reviewed in Tharun and Parker, 1997). Several lines of evidence suggest a role for PABP in maintaining this sequence of events during the degradation of mRNA (reviewed in Tharun and Parker, 1997).

Arabidopsis encodes a family of differentially expressed PABP genes (Belostotsky and Meagher, 1993; Hilson *et al*., 1993). The complexity of this gene family was further demonstrated by the high degree of sequence divergence among these genes and their protein products. *Arabidopsis* PAB5 was able to restore viability to a yeast *pab1* null mutant strain. Using this rescued yeast strain, it was found that PAB5 was able to complement translation initiation and poly (A) tail shortening functions of yeast PAB1. However, the ability to inhibit decapping observed for yeast PAB1 was not complemented by PAB5 as it failed to restore the linkage between deadenylation and decapping (Belostotsky and Meagher, 1996). Based on these observations, we hypothesized that functional differences exist among the members of the PABP gene family in *Arabidopsis*. To test this hypothesis and to further understand the *Arabidopsis* PABP gene family, we have characterized the functional properties of a highly divergent member of this family, *PAB2*.

The presence of multiple PABP genes could complicate the functional analysis of *PAB2* in *Arabidopsis*. This difficulty is compounded by the lack of *Arabidopsis* mutants in *PAB2* and the tissue specific expression of *PAB2* in several different organs (Palanivelu *et al*., 2000). In this study, therefore, we used a yeast *pab1* deleted strain rescued by the expression of PAB2 to perform functional analysis of PAB2. We demonstrate that, similar to yeast PAB1 and *Arabidopsis* PAB5, PAB2 can at least partially substitute for yeast PAB1 in its poly (A) tail shortening and translation initiation functions. However, unlike PAB5, PAB2 can complement the inhibitor of decapping function of yeast PAB1. These results demonstrate that yeast PAB1 functions are conserved in *Arabidopsis* PAB2 and strongly suggests that functional differences and similarities exist between *Arabidopsis* PAB2 and PAB5 proteins.

Results

Phylogenetic relationship among Arabidopsis *PAB proteins*

PAB2 sequences contain all known structural features that are present in a typical PAB protein (Hilson *et al*., 1993). To understand the evolutionary relationships of PAB2 to other *Arabidopsis* PABPs and to PABP from other organisms, we constructed a neighbor-joining tree (NBJ) using complete PAB protein sequences. From this analysis (shown in Figure 1), it is clear that plant PABPs are no more highly diverged from ancestral PABP in other kingdoms than are animal or fungal PABPs. Thus, the plant sequences have

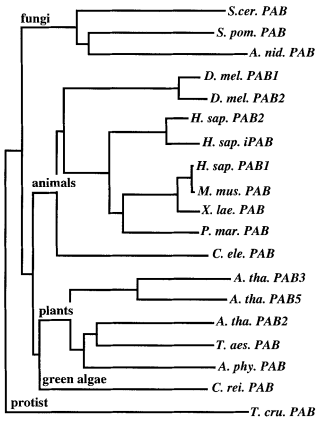

Figure 1. Phylogenetic relationships of PAB proteins from fungi, animals, plants and protists.
The complete amino acid sequence of each PABP were obtained from GenBank and aligned. Distance matrices obtained from this comparison were then used to build a neighbor-joining tree. Branch lengths are scaled as estimated by the neighbor-joining program, are rate sensitive and proportional to genetic distance. Genus species abbreviations are as follows: *S. cer., Saccharomyces cerevisiae*; *S. pom., S. pombe*; *A. nid., Aspergillus nidulans*; *D. mel., Drosophila melanogaster*; *H. sap., Homo sapiens*; *M. mus., Musculus musculus*; *X. lae., Xenopus laevis*; *P. mar., Petromyzon marinus*; *C. ele., Caenorhabiditis elegans*; *A. tha., Arabidopsis thaliana*; *T. aes., Triticum aestivum*; *A. phy., Anemia phylliditis*; *C. rei., Chlamydomonas reinhardii*; *T. cru., Trypanosoma cruzi.*

evolved at rates typical of other PABPs, but are very divergent from each other. Although the plant PAB proteins are highly divergent from yeast PAB1 (*S.cer.PAB*), wheat (*T.aes.PAB*) (Le *et al.*, 1997a) and *Arabidopsis (A.tha.PAB5)* (Belostotsky and Meagher, 1996), proteins have both been shown to functionally complement yeast PAB1. Thus, in spite of the sequence divergence, there is sufficient functional conservation among PAB proteins to complement those yeast functions required for viability. Interestingly, two of the reproductive tissue-specific (Belostotsky and Meagher, 1993) *Arabidopsis* PAB proteins, PAB3 and PAB5, formed a separate clade, whereas PAB2 grouped with wheat PAB, suggesting that PAB2 shares a more recent ancestry with this wheat PAB than to other *Arabidopsis* PABPs. These results strongly suggest that PAB2 is highly divergent in time and sequence from PAB5 and PAB3. The fact that yeast PAB1 is a multi-functional protein and that *Arabidopsis* PABPs exhibit high sequence divergence raise the possibility that functional differences exist among *Arabidopsis* PABPs. Such a possibility could be tested by comparing the functional analysis of PAB2 in yeast with what has been previously reported for *Arabidopsis* PAB5 (Belostotsky and Meagher, 1996).

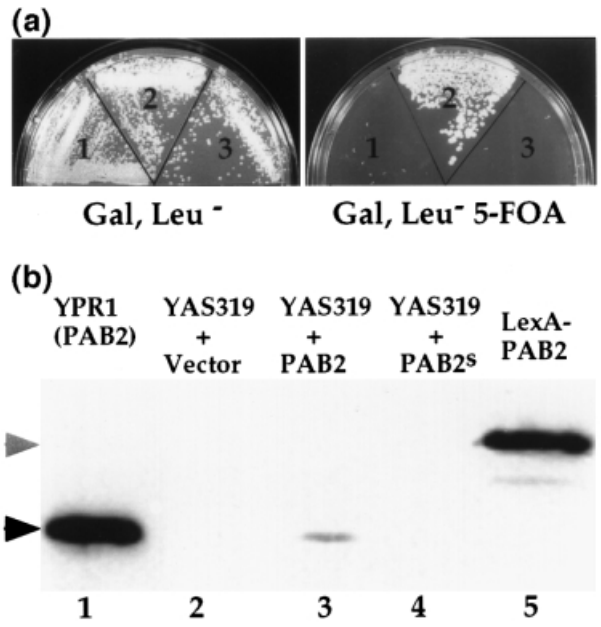

Figure 2. PAB2 restores viability to yeast *pab1*⁻ strain.
(a) YAS319 with pYEp51 vector (1) or pYEp-51/*PAB2* (2) or pYEp51-*PAB2*^{S} (3) were streaked out on a galactose medium without leucine and with (right) or without 5-FOA (left). Successful complementation on 5-FOA was achieved only by the yeast strain containing pYEp-51/*PAB2* (2).
(b) Western blot analysis with ~10 µg of total protein extracts from the indicated strains were probed with PAB2-NT polyclonal antibody. The black arrow indicates the expected size (~69 kDa) of PAB2 protein. LexA-PAB2 indicates the wild-type yeast strain in which LexA-PAB2 fusion protein is expressed. The grey arrow indicates the expected size (~95 kDa) of LexA-PAB2 fusion protein.

PAB2 restores viability to yeast pab1 mutant strain

To perform functional analysis of PAB2, we created a yeast strain in which PAB2 is the only source of PABP. We placed the complete coding region of *PAB2* under the control of a *Gal10* promoter on a *LEU2*-marked, high-copy expression vector (Experimental procedures). This *PAB2* expressing construct was then transformed into YAS 319 yeast strain, which had the chromosomal copy of yeast *PAB1* gene deleted and complemented with the yeast *PAB1* gene on a *URA3*-marked plasmid. The transformants were then streaked on medium containing 5-fluoroorotic acid to

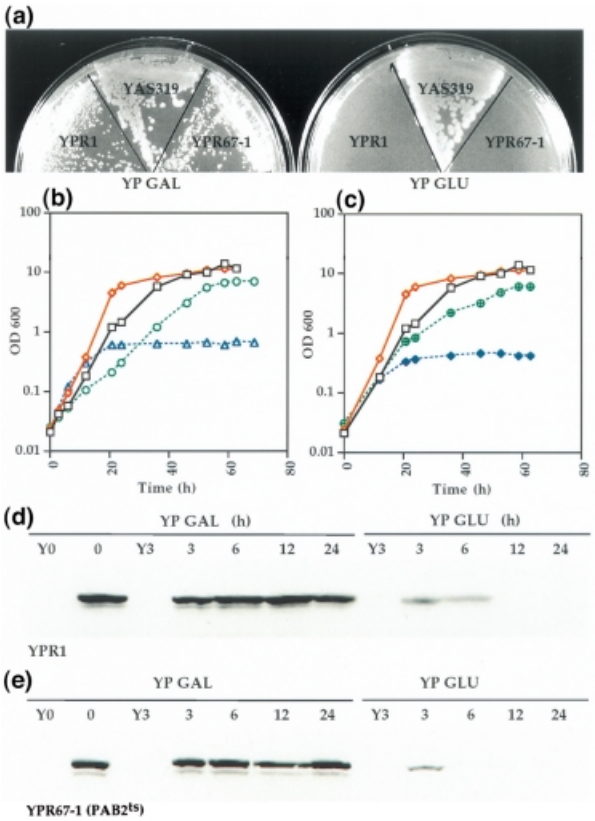

Figure 3. Galactose-dependent growth phenotype of YPR1 and YPR67–1 strains.
(a) Indicated strains were streaked either on rich galactose medium (YPGAL) or rich glucose medium (YPGLU).
(b) Growth curves analysis of YAS319 and YPR1 (PAB2) in glucose and galactose medium. The strains were grown in galactose for two doublings and then shifted (0 h) either to galactose (YAS319: solid lines with squares, YPR1: dashed lines with circle) or to glucose (YAS319: solid line with diamonds, YPR1: dashed lines with triangles).
(c) Growth curve analysis of YAS319 and YPR67-1 (PAB2^{ts}) strains in glucose and galactose medium. The strains were grown in galactose for two doublings and then shifted (0 h) either to galactose (YPR67-1: dashed with filled circles) or glucose (YPR67-1: dashed with filled diamonds). Growth data for YAS319 used in this panel have been reproduced from (b).
(d,e) Western blot analysis of PAB2 protein expression in ~10 µg extracts from YPR1 and YPR67-1, respectively. All the strains were grown in galactose and then shifted (0 h) either to glucose or galactose for the indicated hours. In each of the Western blots, extracts from YAS319 strain grown for 0 h (Y0) or 3 h (Y3) were included as negative controls.

perform a plasmid shuffle assay (Sikorski and Boeke, 1991), which selects for cells that have lost *URA3* containing plasmid expressing the only functional yeast *PAB1* gene. The results of a typical experiment are shown in Figure 2(a). YAS319 with the Leu2 vector alone (sector 1, Figure 2a) or the Leu2 vector expressing the *PAB2* coding sequence with a stop codon ($PAB2^S$) in the 89th amino acid position (sector 3, Figure 2a) were unable to support growth on 5-FOA (see Experimental procedures). In contrast, plasmid expressing unaltered *PAB2* (sector 2, Figure 2a) was able to restore viability to the yeast *pab1* strain. In addition, the cells that grew on 5-FOA plate (referred to as YPR1) were unable to grow on medium lacking uracil, confirming the absence of *URA3* marked plasmid carrying the yeast *PAB1* gene. Western blot analysis revealed that no full length PAB2 protein could be detected either in cells transformed with vector or $PAB2^S$ (Figure 2b, lanes 2 and 4). However, PAB2 protein was detected both in the YAS319 transformed with *PAB2* before 5-FOA selection, and in YPR1 (Figure 2b, lanes 3 and 1, respectively). These results indicate that strains which lack full length PAB2 protein were unable to complement the loss of yeast *PAB1*. A wild-type yeast strain with *LexA-PAB2* fusion protein was included as a control to confirm the validity of the immune assay. The parent strain YAS319 with an empty vector was included to demonstrate that neither yeast PAB1 nor any other yeast protein of the same size as PAB2 could be detected by anti-PAB2 antibody.

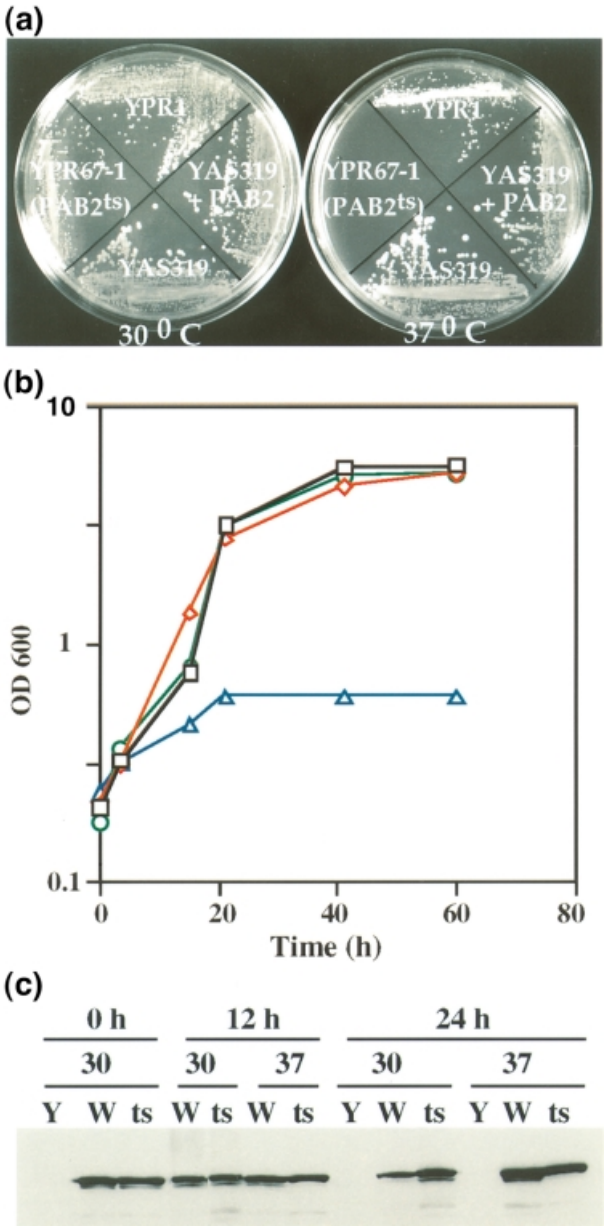

Figure 4. Temperature sensitive growth phenotype of YPR67-1 strain.
(a) Indicated strains were streaked on rich galactose medium (YPGAL) and incubated at 30°C (left) or 37°C (right).
(b) Growth curve of YPR1 (PAB2) and YPR67-1 ($PAB2^{ts}$) strains. Both strains were grown in rich galactose medium at 30°C for two doublings and then shifted (0 h) to galactose medium and incubated either at 30°C (YPR1: diamonds, YPR67-1: squares) or 37°C (YPR1: circles, YPR67-1: triangles).
(c) Western blot analysis of PAB2 protein expression in approximately 10 μg of extracts from YPR1 and YPR67-1 grown in galactose medium at 30°C and then shifted (0 h) to galactose medium at 30°C or 37°C for the indicated hours. YAS319 (Y) extracts were included as negative controls. W = PAB2 wild-type protein in YPR1 and ts = PAB2 ts protein in YPR67-1.

To confirm further the requirement of PAB2 protein for the growth of YPR1, we eliminated PAB2 expression using the glucose-repressible *GAL10* promoter that drives PAB2 expression. YPR1 was streaked in rich medium containing either glucose or galactose alongside of YAS319. As can be seen in Figure 3(a), although YAS319 was able to grow in both media, YPR1 was able to grow only in galactose and not in glucose. The failure of YPR1 to grow in glucose was also observed in growth curves (Figure 3b) in which the growth of YPR1 was arrested after approximately five cell doublings. Consistent with this failure to grow in glucose medium, Western blot analysis revealed a gradual decrease in PAB2 protein levels when YPR1 was shifted from galactose to glucose, but this decrease did not occur during continued growth in galactose (Figure 3d). These results strongly demonstrate the requirement of PAB2 expression for YPR1 growth. By-pass suppressors of yeast *pab1* deletion occur at high frequency (Sachs and Davis, 1989). Galactose-dependent growth of YPR1 eliminates the possibility that the survivors on 5-FOA plate were by-pass suppressors of yeast *pab1* deletion.

Isolation and characterization of a temperature-sensitive allele of PAB2

Functional analysis of PAB2 could be greatly facilitated by the fact that its expression is controlled by a carbon source repressible promoter, *Gal10*. However, growth curve analysis (Figure 3b) revealed that switching from galactose to glucose does not cause a growth arrest until after

approximately five doublings have taken place. A delayed growth arrest after a carbon source switch might interfere with the interpretation of the results from functional analysis. The problem can be circumvented by using conditional lethal alleles (Belostotsky and Meagher, 1996). Hence we constructed temperature sensitive (ts) alleles of *PAB2* in yeast by the random PCR mutagenesis approach (Experimental procedures). One of the ts alleles (*PAB2*^{ts}), expressed from pPR67-1, complemented the yeast *pab1* deletion only at 30°C and not at 37°C, and exhibited a galactose-dependent growth phenotype (Figure 3a,c,e).

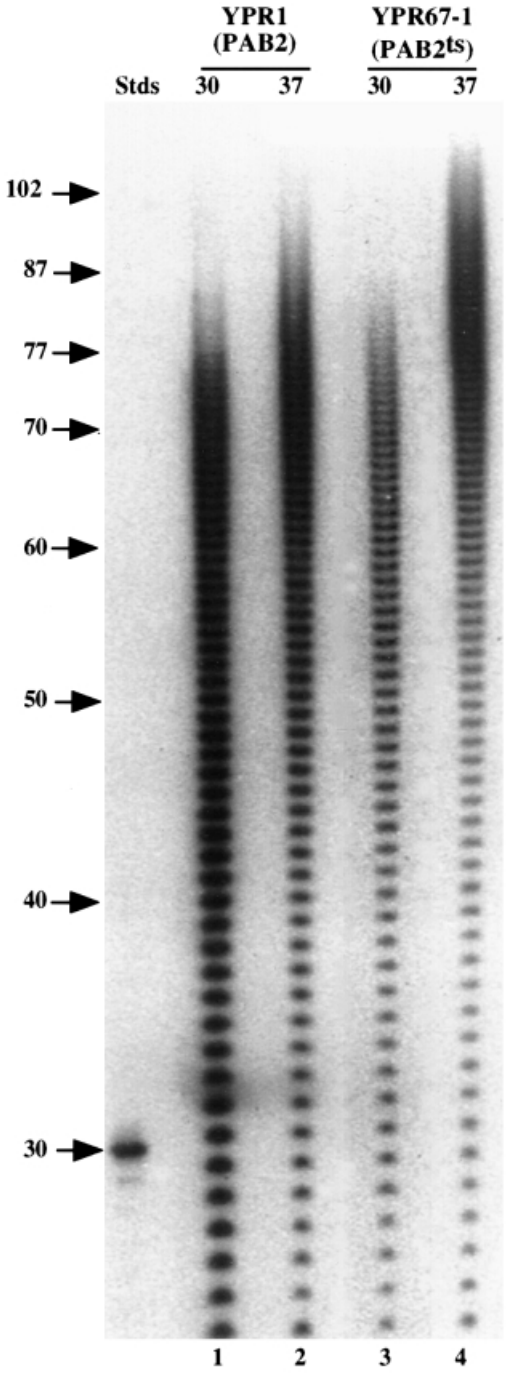

Figure 5. The distribution of poly (A) tail lengths in YPR1 and YPR67-1 strains grown at 30°C and shifted for 12 h to 37°C.
Poly (A) tails were isolated by 3′ end labeling of total RNA from each strain with ^{32}pCp, digestion of all but the poly (A) tails by RNase A and RNase T1, resolved on a 12% polyacrylamide –7M urea gel. Poly (A) tail lengths are indicated in nucleotides. 5′ end-labeled synthetic poly (A) tails of 30 nucleotides long were used as size standards.

Plating and growth curve analysis demonstrated that the yeast strains expressing the PAB2^{ts} mutant protein YPR67-1 could grow at almost the same rate as YPR1 at the permissive temperature (30°C), but were unable to grow at the non-permissive temperature (37°C) (Figure 4a). In addition, complete growth arrest was evident even after two doublings (Figure 4b). As in YPR1, shifting YPR67-1 from galactose to glucose also resulted in the decrease of full length PAB2 protein expression (Figure 3e). Western analysis also revealed that after the shift to glucose the PAB2^{ts} protein disappeared rapidly and was approximately half as stable as the wild-type protein (compare Figure 3d and e), even when grown at permissive temperature. This raised the possibility that PAB2^{ts} protein in the YPR67-1 strain could be even more unstable at higher temperatures and that the physical loss of PAB2^{ts} protein could be the cause of its temperature-sensitive growth phenotype. However, Western analysis that compared the levels of PAB2 proteins in YPR67-1 cells grown, respectively, in galactose at 30°C and 37°C did not support this proposal. In fact, PAB2^{ts} protein was present even 24 h after the shift to the non-permissive temperature suggesting that increase in temperature did not significantly alter its stability (Figure 4c, compare PAB2 protein levels in ts strains at 30°C and 37°C). Based on these Western results it can be hypothesized that the loss of functional PAB2^{ts} protein is the basis for the temperature-sensitive growth phenotype of YPR67-1.

Inactivation of PAB2 leads to accumulation of longer poly (A) tails

Depletion or inactivation of yeast PAB1 leads to the accumulation of longer poly (A) tails, and resolvable maximal lengths increase from ~67 nucleotides to 85–95 nucleotides (Sachs and Davis, 1989). In addition, elimination of the yeast PAB-dependent PAN complex also leads to accumulation of similar longer poly (A) tails (Brown *et al.*, 1996). Hence, we explored the ability of PAB2 to interact with the yeast PAN system by evaluating the poly (A) tail lengths of steady-state mRNAs from yeast strains with normally expressed PAB2 and inactivated PAB2. Total RNA was isolated from YPR1 and YPR67-1 strains grown at 30°C and then shifted either to 30°C or 37°C for 12 h. The total RNA was end-labeled with (^{32}P) pCp and RNA ligase, followed by RNase T1 and RNase A digestion. The remaining intact, labeled poly (A) tails were resolved on

polyacrylamide gels. The maximal poly (A) tail length in YPR1 and YPR67-1 grown at 30°C was ~77 nucleotides (Figure 5, lanes 1 and 3 and Table 1), about 10–15 nucleotides less than the resolvable maximal length in a yeast *pab1* strain (Brown *et al.*, 1996). Quantifying the distribution of all poly (A) tails 25 nucleotides, or those greater in length, revealed that 8–11% of the tails were 70 nucleotides in length or greater in YPR1 and YPR67-1 grown at a permissive temperature (Figure 5, lane 1 and 3 and Table 1). In comparison, 18% of the tails in this range, in a yeast strain which lacks a PAB1-dependent PAN system, comprised of PAN2 and PAN3 and 7% in a wild-type yeast strain (Brown *et al.*, 1996). Thus, the resolvable maximal length and size distribution of poly (A) tails of YPR1 and YPR67-1 strains were lower when compared to yeast strains in which yeast PAB1 grown at 30°C is inactivated, suggesting that PAB2 complements, at least partially, the poly (A) tail metabolism function of yeast PAB1.

A significant increase occurred in the resolvable maximal length of poly (A) tails and the percentage of poly (A) tails greater than 70 nucleotides when YPR67-1 and YPR1 strains grown at 37°C were compared (Figure 5, lane 2 and lane 4, Table 1). This increase demonstrated that inactivation of PAB2 led to the accumulation of longer poly (A) tails, confirming the ability of PAB2 to participate in the poly (A) tail metabolism function of yeast PAB1. The comparison of YPR67-1 grown at 37°C with YPR1 was essential to understanding the effect of PAB2 inactivation on poly(A) tail length control because increased temperature by itself caused some increase in the maximal poly (A) tail length and percentage of poly (A) tails greater than 70 nucleotides in YPR1 (Figure 5, compare lanes 1 and 2 and Table 1). A similar effect of temperature on poly (A) tail length increase was reported for the yeast *PAB1* alleles *pab1-F364* and *pab1-F364L* (Sachs and Davis, 1989).

Table 1. Poly (A) tail length profiles of total mRNAs in YPR1 and YPR-67-1 strains

Strain	Growth temperature (°C)	Maximal resolved poly (A) tail length (nucleotides)	Tails greater than 70 nucleotides (%)[a]
YPR1	30	77	8
YPR1	37	87	23
YPR67-1	30	77	10
YPR67-1	37	102	55

[a]The percentage of tails greater than 70 nucleotides were quantified using Molecular Dynamics Phosphorimager and ImageQuant software.

Inactivation of PAB2 leads to a block in translation initiation

The molecular phenotype of a translation initiation block is an increase in the amount of 80S monosomes concomitant with a decrease in the amount of polysomes (Moldave, 1985). Because it was previously shown that yeast PAB1 is involved in translation initiation, we used YPR67-1 grown at 30°C and 37°C to explore the role of PAB2 in translation initiation. The monosome : polysome ratios of YPR1 and YPR67-1 strains grown for 12 h at 30°C were comparable to that of YAS319 grown under similar conditions (Figure 6, top profiles), suggesting that PAB2 can play a role in translation initiation and restores this function in the absence of yeast PAB1. Further evidence for the involvement of PAB2 in translation initiation was the significant change in the monosome : polysome ratio in YPR67-1 shifted to 37°C, compared to the observed ratio in the same strain grown at 30°C (Figure 6, compare top and bottom profiles of YPR67-1) or compared to the ratios of YAS319 and YPR1 grown at 37°C. Thus, inactivation of $PAB2^{ts}$ protein resulted in a polysomal profile that was typical of a block in translation initiation, confirming the ability of PAB2 to participate in translation initiation in yeast.

Poly (A) enhances in vitro *binding of PAB2 to* Arabidopsis *eIF-iso4G*

The mechanistic understanding of yeast PAB1's involvement in translation initiation has come from the finding

Table 2. Maximal poly (A) tail lengths of reporter mRNA decay intermediates in different yeast strains

Strain	Reporter mRNA	Figure	Maximal resolvable poly (A) tail length (nt) without oligodT*	Maximal resolvable poly (A) tail length (nt) with oligo dT*
YRP840	PGK1pG	8B	15	10
YRP881	PGK1pG	8B	75	10
YPR1	PGK1pG	8B	**25**	10
YRP840	MFA2pG	8C	20	10
YRP881	MFA2pG	8C	75	10
YPR1	MFA2pG	8C	**40**	10

*As observed in Figure 8(b,c).

that it interacts with the initiation factor eIF4G in a poly (A)-dependent manner to recruit the 40S ribosomal subunit to mRNA (reviewed in Sachs *et al.*, 1997). The involvement of PAB2 in translation initiation prompted us to explore if PAB2 physically interacts with the *Arabidopsis* homologue of eIF4G. We overexpressed the amino terminal half of *Arabidopsis* eIF-iso4G (amino acids 1–500) as a GST fusion protein in *Escherichia coli*. GST or GST-fusion proteins immobilized on glutathione sepharose beads were then incubated with ^{35}S-labeled, *in vitro* translated PAB2 protein in the presence or absence of poly (A) ribonucleotides. After extensive washing, bound PAB2 was eluted and resolved on a polyacrylamide gel. In the presence of poly (A), there was significant enhancement in the binding of PAB2 to GST-eIF-iso4G, compared to the binding of PAB2 to GST alone (Figure 7, lanes 1 and 2, black arrow). However, in the absence of poly (A), there was no discernible difference in the trace amounts of PAB2 that bound to either GST or GST-eIF-iso4G (Figure 7, lanes 3 and 4). The observed enhancement in binding in the presence of poly (A) was reproducible in independent experiments. Based on these observations we conclude that poly (A) causes an enhancement in the binding of *Arabidopsis* PAB2 to eIF-iso4G *in vitro*. It should be noted that the identity of the ^{35}S-labeled lower molecular weight bands found in the *in vitro* translation of PAB2 gene (Figure 7, lane 5) and the significance of these products binding to GST-eIF-iso 4G are not clear. Further investigation is necessary to find out if all of these lower molecular weight products of PAB2 that do bind eIF-iso4G possess the eIF-iso4G binding domain.

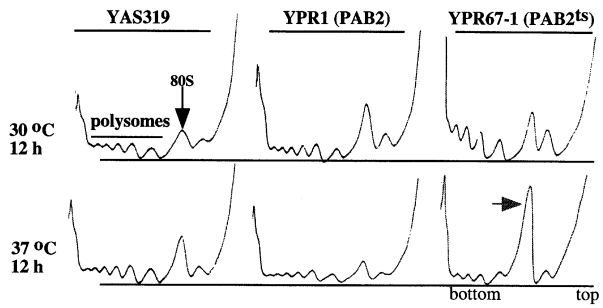

Figure 6. Polysome profile analysis of YPR1 and YPR67-1 yeast strains.
Extracts prepared from indicated strains grown in rich galactose medium (YPGAL) at permissive (30°C) or non-permissive temperatures (37°C) for 12 h were separated on 7–47% sucrose gradients. Gradients were fractionated from the top and scanned at 254 nm. The grey arrow points to the increased accumulation of 80S monosomes of YPR67-1 when grown at 37°C. The following apply to every profile: positions of 80S monosomes (black arrow) and polysomes as indicated in the top left most panel; top and bottom of gradient as indicated in the bottom right panel.

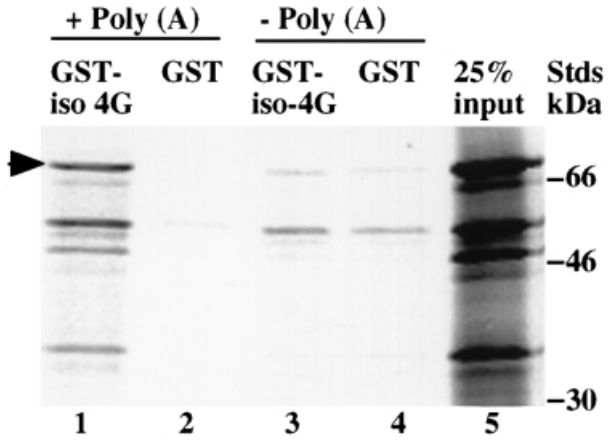

Figure 7. PAB2 binds *in vitro* to *Arabidopsis* iso eIF4G in a poly (A)-dependent manner.
The GST fusion protein containing 1–500 amino acids of eIF-iso4G or GST alone was immobilized on glutathione resin and then incubated with ^{35}S methionine-labeled, *in vitro* translated PAB2 either with or without pre-incubation with poly (A). Subsequent to washing, the bound PAB2 was eluted and ran on 10% SDS-PAGE gel. The gel was dried and exposed to film. The black arrow points to the full length PAB2 protein of expected size (~69 kDa).

PAB2 partially restores the linkage between deadenylation and decapping

The major mRNA degradation pathway in yeast initiates with the removal of poly (A) tails down to 10–12 adenosine residues, followed by decapping (by DCP1) and degradation of the body of the message by 5′-3′ exonuclease, XRN1 (Figure 8a, left, adapted from Tharun and Parker, 1997). This major degradation pathway was elucidated using reporter mRNAs with a poly (G) tract inserted in them to trap decay intermediates which otherwise would be rapidly degraded by XRN1 (Figure 8a, left). When this reporter system was used in the yeast *pab1* strain, 3′ decay intermediates accumulated with long poly (A) tails (Figure 8a, right) suggesting that (i) in the absence of yeast PAB1, decapping occurred prior to deadenylation and the requirement for poly (A) tail removal prior to decapping is bypassed, and (ii) PAB1 is involved in the deadenylation that precedes degradation. In addition, the lethality of *pab1* deletion was suppressed by *xrn1* or *dcp1* deletion, suggesting that PAB1 has an essential function in this pathway (Beelman *et al.*, 1996). The essentiality of PABP involvement in this pathway was not consistent with the observation that *Arabidopsis* PAB5 was unable to restore the linkage between deadenylation and decapping, although it restored viability to a yeast *pab1* strain (Belostotsky and Meagher, 1996). In this context, we wanted to explore the ability of PAB2 to restore the linkage between deadenylation and decapping.

We transformed mRNA decay reporter plasmids encoding highly stable *PGK1* or highly unstable *MFA2* mRNAs with a poly (G) insert (Caponigro and Parker, 1995) into YPR1. Northern analysis was performed on RNase H-oligo (dT) treated and untreated samples of total RNA from

these strains as described by Tanzer and Meagher (1994) and Caponigro and Parker (1995) to assess the poly (A) tail length of full length and decay intermediates of reporter mRNAs. In each case, the poly (A) tail length of decay intermediates was compared with those of YRP840 (wild-type) and YRP881 (bypass suppressor of *pab1* deletion) strains (Caponigro and Parker, 1995). As has been reported previously (Caponigro and Parker, 1995) for YRP840, decay intermediates accumulated with very short poly (A) tails up to 15–20 nucleotides (compare lanes 1 and 2 in Figure 8b and c, and Table 2) and for YRP881 decay intermediates accumulated with long poly (A) tails up to 75 nucleotides (compare lanes 3 and 4 in Figure 8b,c). In YPR1, however, the decay intermediates accumulated with poly (A) tails whose resolvable maximal length was approximately 25 nucleotides in the case of *PGK1* (Figure 8b, lanes 5 and 6), and approximately 40 nucleotides in the case of *MFA2* (Figure 8c, lanes 7 and 8, and Table 2). Even upon overexposure, the maximal poly (A) tail length of the decay intermediates in YPR1 was still shorter than those of YRP881 (data not shown). The accumulation of decay intermediates with shorter tails in YPR1 relative to YRP881 demonstrates that *Arabidopsis* PAB2 can restore, at least partially, the linkage between deadenylation and decapping in yeast. In contrast, *Arabidopsis* PAB5 was indistinguishable from the bypass suppressor YRP881 in this assay (Belostotsky and Meagher, 1996). The experiments on PAB5 were carried out in identical genetic backgrounds as that of YPR1.

Our data using the *PGK1* and *MFA2* mRNA decay reporter plasmids in YPR1 demonstrate that PAB2 can partially restore the linkage between deadenylation and decapping in a yeast PAB1 deficient strain. However, two minor observations need to be explored. First, the Northern analysis using *MFA2/pG* revealed that the poly (A) tail length of the shortest decay intermediates in YPR1 is longer than those found in YRP840 and YRP881 (Figure 8c, compare DI in lanes 7 with those in lanes 1 and 3). Second, the ratio of degradation intermediates to full-length messages was lower in YPR1 compared to YRP840 and YRP881. These observations in YPR1 were reproducible in independent experiments (data not shown) and were found only with *MFA2/pG* and not with *PGK1/pG* reporter constructs. Therefore we set out to determine if these two

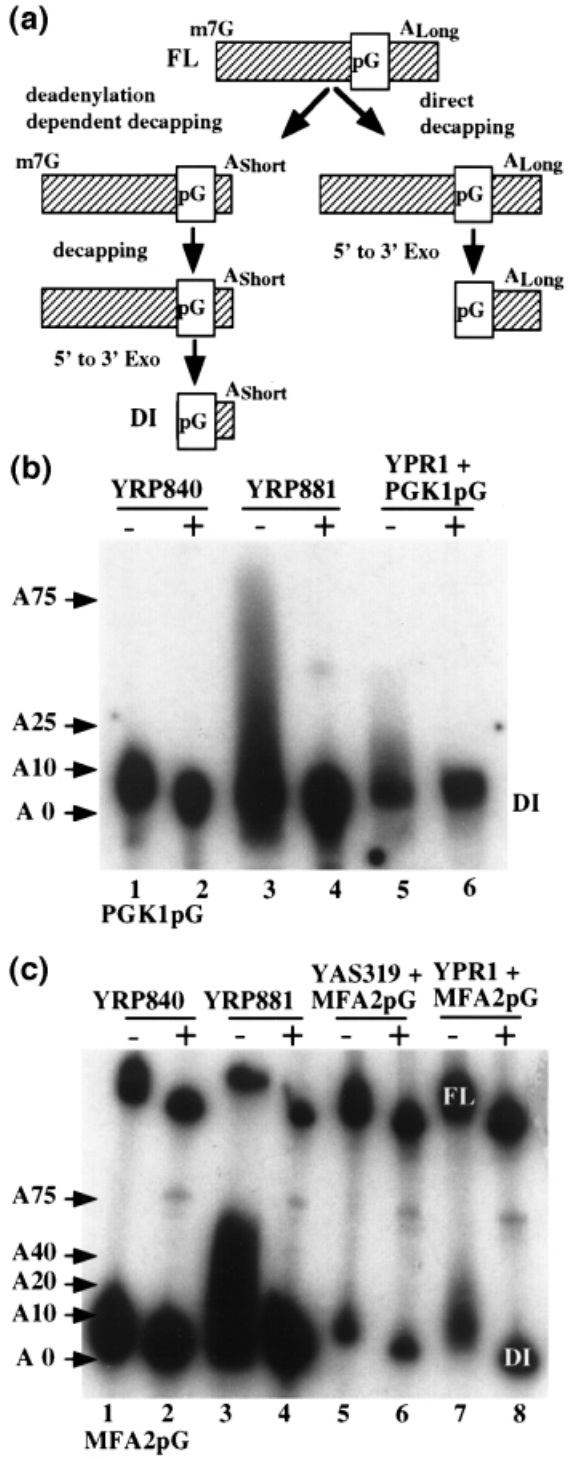

Figure 8. Partial restoration of linkage between deadenylation and decapping by PAB2 in yeast.

(a) Diagram of the major degradation pathway of mRNAs in yeast. The decay of mRNAs initiates with deadenylation followed by decapping and 5′–3′ exonucleolytic degradation in wild-type yeast strains (left). In *pab1* deficient strains, premature decapping occurs even before mRNAs are deadenylated (right). The insertion of poly (G) tracts (pG) slows down the 5′–3′ exonuclease (exo), leading to the accumulation of intermediates either with short poly (A) tails (AShort) in wild-type strains or long poly (A) tails (Along) in *pab1* deficient strains.

(b,c) Total RNA from indicated strains treated with oligo d(T) and incubated with (+) or without (–) Rnase H. The RNA was then resolved on a polyacrylamide gel and probed with either (b) *MFA2*/pG specific oligonucleotide ORP 140 or (c) *PGK1*/pG specific oligonucleotide ORP 141 (Caponigro and Parker, 1995). FL-full length *PGK1*/pG or *MFA2*/pG mRNA and DI–decay intermediates. Poly (A) tail lengths of the decay intermediates indicated on the left side of (b and c) were estimated using ethidium bromide stained RNA standard bands (GIBCO BRL).

changes are due to PAB2 or the background strain differences between YAS319 and YRP840 (YRP881 has the same background as that of YRP840). We transformed the *MFA2/pG* reporter construct into YAS319, from which YPR1 was derived. As evident from Figure 8(c) (compare lanes 5 with lanes 1, 3 and 7), the length of the lowest decay intermediate and the ratio of decay intermediates to full-length messages in YAS319 was essentially the same as observed in YPR1, confirming that these two distinctions from the controls are due to the background strain differences.

Discussion

Our data demonstrating that *Arabidopsis* PAB2 can restore viability to a yeast *pab1* mutant strain strongly argues that it encodes for a functional poly (A) binding protein. This is consistent with the predicted identity of PAB2 based on protein sequence comparison (Hilson *et al.*, 1993). Although PAB2 was able to complement yeast PAB1 loss (Figure 2a), it was able to do so only when expressed from a high-copy vector and not when expressed from a low-copy vector (see Experimental procedures). In support of this observation, Western blot analysis was able to detect PAB2 protein when expressed from a high-copy vector (Figure 2b, lane 3), but not from a low-copy vector (data not shown), upon transformation into YAS319 yeast strain. Surprisingly, only low levels of PAB2 protein were detected in yeast strain YAS319 expressing PAB2 from the high copy vector, in contrast to the YPR1 strain in which no yeast PAB1 is present (Figure 2b, compare lanes 1 and 3). This suggests that the low levels of PAB2 could result from the negative regulation of PAB2 expression by yeast PAB1. Only the protein coding region of PAB2 was expressed from the gal promoters of the high-copy and low copy vectors. Therefore, the poly (A) rich regions in the 5′UTR of PABP mRNAs from different species, which have been implicated in the autoregulation of PABP expression, cannot account for this negative regulation of PAB2 (de Melo Neto *et al.*, 1995).

Although PAB2 restores viability to a yeast *pab1*-deleted strain, the growth rate analysis (Figure 3b) indicates that YPR1 has a generation time of approximately 6 h in galactose medium compared to approximately 3 h of a wild-type strain under similar conditions. Incomplete functional complementation could be one of the reasons PAB2 is unable to restore wild-type growth rates. In fact, our results show that PAB2 only partially complements the poly (A) tail length control and restoration of linkage between deadenylation and decapping functions of yeast PAB1. Alternatively, levels of PAB2 protein in the YPR1 strain could simply have been lower than the levels required to achieve complete complementation, thus resulting in a slower growth rate.

Higher plants contain two forms of eIF4G, namely eIF-4G and eIF-iso4G. Both exhibit similar activities (Browning *et al.*, 1987) and both forms are distant homologues of yeast eIF-4G. We found that poly (A) enhances PAB2 binding *in vitro* to the *Arabidopsis* initiation factor eIF-iso4G (Figure 7). This interaction is consistent with PAB2's involvement in translation initiation and with the finding that poly (A) bound yeast PAB1 interacts with eIF-4G and stimulates translation *in vitro* (Kessler and Sachs, 1998; Tarun and Sachs, 1996; Tarun *et al.*, 1997). This experiment did not distinguish between a direct binding to eIF-4G or tethering through a common interaction with poly$(A)_{50–200}$. However, a tethered interaction through poly(A) strong enough to affinity purify PAB2 bound to eIF-iso4G is unlikely, because the RNA binding of wheat eIF-iso4G strongly favours G-rich sequences (Kim *et al.*, 1999). The poly (A) mediated enhancement in binding of PAB2 eIF-iso4G agrees well with the finding that poly (A) serves as a platform on the mRNA to which PABP can perform its functions when bound (Coller *et al.*, 1998), and may explain why the excess of PABP (compared to poly (A)) in a cell does not interfere with its functions (Gorlach *et al.*, 1994). Our conclusion that poly (A) causes enhanced interaction between PAB2 and eIF-iso4G is different from that of another study, which found that poly (A) neither enhances nor decreases the interaction between wheat PABP and wheat eIF-iso4G (Le *et al.*, 1997b). It is possible that our differing conclusions about the requirement of poly (A) are attributable to the differences in the *in vitro* conditions used in the binding assays and/or more likely the functional differences among the diverse plant PAB protein family members in any single plant. Nevertheless, our findings in conjunction with those of Le *et al.* (1997a) showing that wheat PABP interacts with wheat eIF-iso4G suggest that the mechanism by which PABP stimulates translation initiation in higher plants might be similar to that observed in yeast. Only by selective constraint in plants could such a specific interaction be conserved during the billion years since plants and yeast shared a common ancestor.

The conservation of poly (A) tail length control and translation initiation functions in PAB2 is interesting in the context that these functions were also shown to be conserved in PAB5. PAB5 shows extreme sequence divergence and different organ and tissue expression patterns from PAB2 (Belostotsky and Meagher, 1996; Palanivelu *et al.*, 2000). However, unlike yeast PAB1 and *Arabidopsis* PAB2, PAB5 was unable to restore, even partially, the linkage between deadenylation and decapping in yeast. These findings are in agreement with the observation that while yeast PAB1 and *Arabidopsis* PAB2 share a domain (highly conserved but not identical in sequence) required for this function, PAB5 lacks it altogether (J. Coller and M. Wickens, personal commu-

nication). To further dissect the functional differences between PAB2 and PAB5, it might be interesting to compare overall mRNA stability in yeast strains complemented with these two proteins. Although the functional similarities and differences between PAB2 and PAB5 observed in yeast are yet to be demonstrated in *Arabidopsis*, it still strongly suggests that functional specialization exists among *Arabidopsis* PAB proteins and that it could be one of the reasons a large PABP gene family is maintained by the small genome of *Arabidopsis*.

The advantages of *Arabidopsis* possessing PAB proteins with similarities and differences in function are quite conceivable given that it is a multicellular organism with different developmental phases. One possible scenario during post-transcriptional regulation is that when different mRNA substrates have similar functional needs, multiple PABPs that can perform the same function can be recruited to these mRNAs. Alternatively, PABP with differences in function can be recruited at the same time to several mRNA substrates which differ in their functional needs. Such circumstances would require that more than one PABP be expressed in the same cell type. In support of the view that co-expressed isovariants are required, at least four different *Arabidopsis* PABP genes (*PAB1, PAB2, PAB3* and *PAB5*) are expressed in flowers (Belostotsky and Meagher, 1993; Belostotsky and Meagher, 1996; Hilson *et al.*, 1993; Palanivelu *et al.*, 2000). Isovariant dynamics among diverse PABPs could provide an additional level of post-transcriptional control (Meagher *et al.*, 1999). Thus, with overlap and differences in the functions and expression patterns of PABPs, *Arabidopsis* may be well-equipped to mediate complex post-transcriptional regulation of several mRNAs at different stages of plant development.

Experimental procedures

Yeast strains and growth conditions

For complementation experiments, the yeast strain YAS319 (a gift from Alan Sachs; see also Belostotsky and Meagher, 1996) was transformed either with the low-copy plasmid pAS135/PAB2 or the high-copy plasmids pYEp-51/*PAB2* and pPR67-1. pPR67-1 contains the PAB2 ts allele (see below). The transformants were then used to perform a plasmid shuffle assay (Sikorski and Boeke, 1991). Successful complementation with pYEp51/*PAB2* and pPR67-1 yielded the yeast strains YPR1 and YPR67-1, respectively. Yeast strains YRP840 (wild-type yeast strain with MFA2-poly (G) and PGK1-poly (G) reporter system integrated in the genome) and YRP881 (bypass suppressor of *pab1* deletion by a deletion in *spb2* gene and *MFA2*-poly (G) and *PGK1*-poly (G) reporter system integrated in the genome) have been described previously (Caponigro and Parker, 1995). Standard media, growth conditions and techniques for handling yeast were used (Sachs *et al.*, 1987). Two per cent galactose, 2% glucose and 1% raffinose were used wherever these carbon sources are indicated. All media were pH adjusted to 6.6 with sodium hydroxide before autoclaving.

DNA manipulations

The entire coding region of PAB2 was PCR amplified from an *Arabidopsis* flower cDNA library (Invitrogen) using *PAB2*-specific primers (PAB2-1S – 5′CAGCGTCGACCCATGGCGCAGGTTCAACTTCAG 3′ and PAB2-624N – 5′GTGCGTCGACCTCGAGTTAAGAGAGGTTCAAGGAAGC 3′) with engineered *Sal*I sites in them. The PCR product was then subcloned into *Sal*I digested pBluescript (Stratagene) to yield pBS-PAB2. The PAB2 sequence in this clone was verified in its entirety by comparison with previously reported sequences for *PAB2* (Hilson *et al.*, 1993). Subsequently, the *Sal*I fragment from pBS-*PAB2* was subcloned into a yeast low-copy vector with *Gal1* promoter, pAS135 (Sachs and Davis, 1989) and a yeast high-copy vector with *Gal10* promoter, pYEp-51 (Broach *et al.*, 1983) to yield pAS135-*PAB2* and pYEp51-*PAB2* plasmid clones, respectively.

Isolation of temperature sensitive and null alleles of PAB2

The pYEp51/*PAB2* plasmid clone was used as a template to generate temperature sensitive (ts) alleles of *PAB2* by the PCR mutagenesis/*in vivo* recombination procedure of Muhlrad *et al.* (1992). The primers used in PCR reactions were GAL10–290S (5′-GGCTCTTTACATTTCCACAAC-3′) and pYEp51N3 (5′-TTCAGCAGCACATAATGCTAT-3′). The manganese concentration used for PCR mutagenesis was 0.6–0.7 mM. The mutagenized PCR products were transformed into YAS319. The transformants were subsequently used to perform plasmid shuffle assay at a permissive temperature of 30°C. Survivors from this assay were then tested for galactose dependency for their growth. Temperature sensitive strains were identified from these galactose dependent survivors by testing for their inability to grow at 37°C. Plasmid DNA was isolated (one of them is pPR67-1, the PAB2 protein expressed from this plasmid denoted as $PAB2^{ts}$) from the successful ts strains and re-transformed into YAS319 to perform plasmid shuffle assay to confirm plasmid dependency of the ts phenotype. Complete sequencing of $PAB2^{ts}$ coding region in pPR67-1 revealed the following six point mutations that would result in amino acid changes: $Ser^{160} \rightarrow Pro^{160}$; $Asp^{266} \rightarrow Gly^{266}$; $Ser^{274} \rightarrow Pro^{274}$; $Pro^{528} \rightarrow Thr^{528}$; $Asn^{549} \rightarrow Asp^{549}$; $Thr^{577} \rightarrow Ser^{577}$. A *pab2* mutant that would not complement yeast at any temperature (PAB^{s}) has a nonsense suppressor at position 89 creating a null allele.

Computational analysis

PAB protein sequences of different organisms were obtained from GenBank and protein sequence alignment was created using the Pileup program of GCG software. The sequence alignment was then used to build a neighbor-joining tree using PAUP tree building software.

Determination of poly (A) tail length

Total yeast RNA isolated as per Lin *et al.* (1996) was used to perform poly (A) tail length estimation as described in Sachs and Davis (1989), except that we used RNase T1 in addition to RNase A to digest away the RNA apart from the intact poly (A) tails.

Northern analysis and deadenylation assays

The different yeast strains were grown in rich YPgal medium (yeast extract, peptone and galactose) for two doublings until

they reached an OD of 0.6. At this point the cells were harvested and total yeast RNA was isolated by the modified hot phenol procedure as described by Lin *et al.* (1996). Ten μg of total RNA from each of the yeast strains was bound with 100 pmol of oligo d(T) (Pharmacia) and digested with Rnase H. Digested products were resolved on a polyacrylamide–urea (6% and 8 M, respectively) gel, and Northern analysis was performed as described by Tanzer and Meagher (1994) and Caponigro and Parker (1995). Equal loading was confirmed in repeated experiments by staining gels with ethidium bromide. Blots were hybridized with oligonucleotides (ORP140, MFA2/pG specific oligo and ORP141, PGK1/pG specific oligo (Caponigro and Parker, 1995) at 46°C (T_m –14°C).

Western blot analysis

The amino terminal region of PAB2, which is highly divergent from other plant PABPs (data not shown), was chosen to design a Multiple Antigen Peptide (MAP) called PAB2-NT (QLQGQTPNGSTAAVTSAPAT, representing PAB2 codons 5–24). This peptide was used to raise polyclonal antibodies against PAB2 in rabbits, referred to as PAB2-NT antibody. Total proteins were extracted from different yeast strains by spinning down 1.5 OD of cells and the cells were vortexed with 50 μl of 2× Laemelli buffer (.5 M Tris, pH 6.8, 10% SDS, 33% glycerol, 0.1% β-mercaptoethanol and bromophenol blue) for 2 min. The samples were boiled for 5 min and stored in aliquots in –20°C. Ten μg of total protein were loaded in 10% polyacrylamide gels and Western blot analysis was performed using PAB2-NT antibody at a dilution of 1:2000 (approximately 90 μg of antibody/40 ml incubation solution) essentially as described by Palanivelu *et al.* (2000).

Yeast polysome preparation and gradient analysis

Polysomes were prepared from 50 OD of different yeast strains essentially as described by Cigan *et al.* (1991). In each case, 20 A_{260} units were loaded onto the sucrose gradients (7–47%) for analysis. Sucrose gradients were prepared as follows: 5 ml of 47% and 7% sucrose solution were sequentially layered in 20 ml Beckman polyallomer centrifuge tubes and covered with parafilm. The tubes were laid down horizontally for 5 h and then left to stand straight in 4°C overnight to form the gradient. Consistent polysome profiles were obtained using sucrose gradients prepared in this manner (data not shown). The gradients were scanned from top to bottom at 254 nm using a Dual path monitor UV-2 (Pharmacia).

In vitro *binding of PAB2 to* Arabidopis *eIF-iso4G*

The amino terminal half of *Arabidopsis* eIF-iso4G (1–500 amino acids) was cloned into pGEX-2T (Pharmacia) to be expressed as a GST-fusion protein (see Acknowledgements). Overexpressed GST-eIF-iso4G and GST proteins were purified onto glutathione sepharose beads as described previously (Tarun and Sachs, 1996). PAB2 was *in vitro* translated from the pBS-*PAB2* clone (see also *DNA manipulations* in Experimental procedures) in the presence of ^{35}S-methionine using the rabbit reticulocyte based *in vitro* translation system (TnT, Promega). Seven μl of a 50 μl PAB2 translation mixture was incubated with or without 5 μg of poly (A) (50–200 nucleotides, Miles Scientific) at room temperature or 4°C for 15 min. Poly (A) pre-incubated PAB2 was then mixed either with equal amounts (~3.0 μg) of GST-eIF-iso4G or GST proteins and binding studies were performed essentially as described previously (Tarun and Sachs, 1996). Rainbow marker protein standards (Amersham) were run in gels which were used to estimate protein molecular weights indicated in Figure 7.

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