Arabidopsis thaliana class II poly(A)-binding proteins are required for efficient multiplication of turnip mosaic virus

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The poly(A)-binding protein (PABP) is an important translation initiation factor that binds to the polyadenylated 3′ end of mRNA. We have previously shown that PABP2 interacts with the RNA-dependent RNA polymerase (RdRp) and VPg-Pro of turnip mosaic virus (TuMV) within virus-induced vesicles. At least eight PABP isoforms are produced in Arabidopsis thaliana, three of which (PAB2, PAB4 and PAB8) are highly and broadly expressed and probably constitute the bulk of PABP required for cellular functions. Upon TuMV infection, an increase in protein and mRNA expression from PAB2, PAB4 and PAB8 genes was recorded. In vitro binding assays revealed that RdRp and the viral genome-linked protein (VPg-Pro) interact preferentially with PABP2 but are also capable of interaction with one or both of the other class II PABPs (i.e. PABP4 and PAB8). To assess whether PABP is required for potyvirus replication, A. thaliana single and double pab knockouts were isolated and inoculated with TuMV. All lines showed susceptibility to TuMV. However, when precise monitoring of viral RNA accumulation was performed, it was found to be reduced by 2.2- and 3.5-fold in pab2 pab4 and pab2 pab8 mutants, respectively, when compared with wild-type plants. PABP levels were most significantly reduced in the membrane-associated fraction in both of these mutants. TuMV mRNA levels thus correlated with cellular PABP concentrations in these A. thaliana knockout lines. These data provide further support for a role of PABP in potyvirus replication.

INTRODUCTION

The poly(A)-binding protein (PABP) is an abundant translation initiation factor in the cell that binds to the polyadenylated 3′ end of mRNA. Its interaction with the eukaryotic translation initiation factor 4F complex (composed of the eIF4E, eIF4A and eIF4G proteins), which is bound to the 5′ cap structure of the mRNA, results in the formation of a protein bridge that brings the 5′ and 3′ termini of the mRNA into proximity. This leads to a synergistic enhancement of translation (Sachs, 2000; Wells et al., 1998). PABP is also important for stability (Behm-Ansma et al., 2007; Parker & Song, 2004), biogenesis (Amrani et al., 1997; Brown & Sachs, 1998) and nuclear export of cellular mRNAs (Brune et al., 2005).

Multiple PABP isoforms are normally found in animals and plants (Mangus et al., 2003). Eight PABP genes have been identified in Arabidopsis thaliana (Belostotsky, 2003). The corresponding proteins are divided into four classes based on gene expression and similarity. Class II genes (PAB2, PAB4 and PAB8) are highly and broadly expressed and probably encode the bulk of PABP required for cellular functions (Belostotsky, 2003).

In animal cells, PABP is cleaved by proteinases of picornaviruses (Joachims et al., 1999; Kerekatte et al., 1999; Kuyumcu-Martinez et al., 2002, 2004b; Rodriguez Pulido et al., 2007; Zhang et al., 2007), caliciviruses (Kuyumcu-Martinez et al., 2004a) and retroviruses (Alvarez et al., 2006). The resultant cleavage leads to host translational shutdown (Joachims et al., 1999; Kuyumcu-Martinez et al., 2004a; Zhang et al., 2007). Rotaviruses also inhibit PABP functions through an alternative pathway. The binding of the viral non-structural protein 3 to eIF4G prevents PABP from interacting with proteins bound to the 5′ cap and leads to a decrease in the translation of cellular mRNAs (Montero et al., 2006; Piron et al., 1998).

Paradoxically, PABP also appears to be required for efficient viral RNA synthesis/translation of a number of
animal viruses. Accumulating evidence suggests that most RNA viruses adopt a closed-loop conformation for efficient translation and transcription of their genome (for review see Thivierge et al., 2005). This is achieved by recruitment of translation initiation factors at the 5’ and 3’ ends of the viral RNA and often involves PABP. Poliovirus replication requires genome circularization and proceeds through a protein bridge that involves PABP (Herold & Andino, 2001). Interactions between viral internal ribosomal entry site (IRES), eIF4G, PABP and the poly(A) tail are also important for picornavirus cap-independent translation. Disruption of PABP–eIF4G or PABP–poly(A) tail interactions on IRES-driven reporter mRNAs leads to reduced rates of translation (Bradrick et al., 2007; Khaleghpour et al., 2001; Michel et al., 2000; Svitkin et al., 2001). Similar results were also obtained using IRES derived from the Tobacco etch virus (Gallie, 2001; Gallie et al., 1995).

Turnip mosaic virus (TuMV) is a potyvirus (Fauquet et al., 2005). Its positive single-stranded RNA genome of almost 10 kb contains one long open reading frame and bears a viral genome-linked protein (VPG) covalently linked at its 5’ terminus and a poly(A) tail at the 3’ terminus (Nicolas & Laliberté, 1992). Several lines of evidence indicate that components of the eIF4F complex are required for potyvirus replication. Potyviruses interact with eIF4E or its eIF(iso)4E isoforms via VPG-Pro (Schaad et al., 2000; Wittmann et al., 1997) and disruption of the interaction leads to resistance (Léonard et al., 2000). Requirement for eIF4E/4G or eIF(iso)4E/4G isoforms depends on the virus being studied, as A. thaliana knockout plants for eIF(iso)4E or eIF(iso)4G1/2 genes are resistant to TuMV, lettuce mosaic virus and plum pox virus infection, while disruption of eIF4E1 or eIF4G results in resistance to clover yellow vein virus (Decroocq et al., 2006; Duprat et al., 2002; Lellis et al., 2002; Nicolas et al., 2007; Sato et al., 2005). These results were also confirmed in multiple plant species with the identification of eIF4E and eIF4G isoforms as recessive resistance gene products against potyviruses (Gao et al., 2004; Kang et al., 2005; Nicolas et al., 2003; Ruffel et al., 2002, 2005). Together, these results are indicative of an essential link between potyvirus replication and components of the translation initiation complex.

We have recently reported the interaction of A. thaliana PABP2 with the RNA-dependent RNA polymerase (RdRp) and VPG-Pro of TuMV within virus-induced vesicles, where replication probably takes place (Beauchemin & Laliberté, 2007). This suggests that PABP might also be a required factor for potyvirus infection. The present work shows that VPG-Pro and RdRp of TuMV interact not only with PABP2 but also with PABP4 and PABP8 in A. thaliana, which explains why single pab2, pab4 or pab8 knockout plants were all susceptible to TuMV infection. However, further depletion of PABP in double-knockout plants resulted in reduced TuMV RNA accumulation. These data support a role for PABP in potyvirus infection.

METHODS

Bacterial expression and purification of recombinant proteins in Escherichia coli. For construction of the vector coding for the fusion of A. thaliana PABP4 (At2g3350; GenPept accession number NP_179916) and PABP8 (At1g9760; GenPept accession number NP_564554) with the N terminal region of glutathione S-transferase (GST), sequences from the Salk, Stanford & Plant Gene Expression Center (SSP) clones U10212 and U15101 (Yamada et al., 2003) were amplified by using PCR with primers PABP4–BamHI and PABP4–NotI, or PABP8–BamHI and PABP8–NotI (see Supplementary Table S1, available in JGV Online). The amplified fragments were digested with BamHI/NotI and cloned into similarly digested pGEX-6P1 (GE Healthcare) and used for purification as described for PABP2-GST (Dufresne et al., 2008). TuMV VPG-Pro and histidine-tagged RdRp were purified as previously described by Dufresne et al. (2008) and Menard et al. (1995).

RdRp and VPG-Pro binding assays with PABP. RdRp or VPG-Pro binding assays were performed as previously described by Dufresne et al. (2008) with GST-PABP2, GST-PABP4 or GST-PABP8 proteins. Detection of retained protein was achieved with a mouse monoclonal (Novagen) or polyclonal (Molecular Probes) antibody.

Isolation of single and double pab mutants. PAB2 (SALK_026293), PAB4 (SALK_113383) and PAB8 (SALK_221660) T-DNA insertion lines were obtained from the SALK T-DNA collection (Alonso et al., 2003). pab homoygous mutant plants were identified by PCR using a T-DNA left border primer and gene-specific primers (see Supplementary Table S1, available in JGV Online).

pab2 pab4 and pab4 pab8 double mutants were obtained by crossing the pab4 null mutant with pab2 or pab8 mutants; pab2 pab8 mutants were obtained by crossing the pab8 mutant line with a pab2 mutant.

TuMV inoculation of A. thaliana. Rosette leaves of 3-week-old A. thaliana plants were inoculated as previously described by Dufresne et al. (2008).

RNA isolation and RT-PCR amplification. Total RNA was isolated from 3-week-old uninfected, mock-inoculated or TuMV-infected plants using the RNAeasy plant Mini kit (Qiagen). RNA aliquots (1 μg) were reverse-transcribed to cDNA using the Omniscript RT kit (Qiagen) and used for PCR. The ACT2 (ACT2) (At3g18780; GenBank accession number NM_180280) mRNA was used as positive control for amplification. Primers are provided in Supplementary Table S1.

Quantitative real-time RT-PCR analysis (qRT-PCR). qRT-PCR was performed using SYBr Green MasterMix (Stratagene). Relative amounts of all mRNAs were calculated from threshold cycle values. The ACT2 reference gene was used for normalization and differences in PCR efficiency were taken into account according to the formula:

\[ (E_{target})_{ACTtarget(control-sample)} / (E_{target})_{ACTcontrol-sample} \]

(Pfaffl, 2001). RNA samples for each genotype were extracted from three different pools of four different plants and qRT-PCR was repeated twice. Primers CP-F (5’-TGGCTGTATTACGACGTGCG-3’) and CP-R (5’-CTGCTAAATGTGGGTTTGG-3’) were used for TuMV detection; the other primers are provided in Supplementary Table S1.

Protein extraction and membrane fractionation. Protein extraction and membrane fractionation were performed as previously described on uninfected, mock inoculated and TuMV-infected A. thaliana (Dufresne et al., 2008). Proteins were separated on an 11 % polyacrylamide SDS gel (Laemmli, 1970) and analysed by immuno-blotting as previously described (Dufresne et al., 2008).
Statistical analyses. PABP densitometric levels on immunoblots were compared using Student’s t-test. Data from ELISA binding assays were analysed using one-way ANOVA and Tukey’s test using SAS 9.0 software. Statistical analyses of qRT-PCR were conducted using REST MCS relative expression software (Pfaffl et al., 2002).

RESULTS

Effect of TuMV infection on PABP2, PABP4 and PABP8 protein and mRNA expression

The level of functional PABP is altered following infection by certain animal viruses (Joachims et al., 1999; Kuyumcu-Martinez et al., 2004b). Analysis of TuMV-infected Brassica perviridis (Beauchemin & Laliberté, 2007) indicated that PABP cellular levels were increased when compared with mock-inoculated plants. In order to test if this was also the case in TuMV-infected Arabidopsis thaliana, leaves were homogenized and nuclei, chloroplasts and cell wall debris were removed by centrifugation at 3700 g. Soluble proteins were then separated from membrane-associated proteins by centrifugation at 27 000 g and resuspended in the same volume to allow quantitative evaluation. Total, soluble (S30) and membrane-associated (P30) proteins were separated by SDS-PAGE and subjected to immunoblot analysis with a rabbit serum raised against recombinant AtPAB2. This serum detects A. thaliana PABP2 (68.7 kDa), PABP4 (71.7 kDa) and PABP8 (72.8 kDa). Due to a similar molecular mass, PABP4 cannot be distinguished from PABP8 using one-dimensional SDS-PAGE (Figs 1a and 3c). This serum recognized recombinant GST-tagged PABP2, PABP4 and PABP8 equally well (data not shown). Therefore, it is likely that the intensity of the bands reflects the abundance of all three isoforms in A. thaliana. Fig. 1 shows that a higher level of PABP2 was present in TuMV-infected extracts. Densitometric analysis indicated a 1.37-fold (± SEM, 0.03) increase in total PABP levels (Student’s t-test, P>0.0001, n=3). In both healthy and infected extracts, UDP-glucose pyrophosphorylase (UGPase), which is a marker for the cytoplasm, was detected only in soluble and total (data not shown) protein extracts, which indicates the absence of cytoplasmic protein contamination in the membrane-enriched fraction. In contrast to the predominantly cytosolic PABP detected in healthy B. perviridis (Beauchemin & Laliberté, 2007; Dufresne et al., 2008), we found that class II PABP isoforms were abundant in both S30 and P30 fractions in healthy and TuMV-infected A. thaliana Col-0.

To assess whether the higher level of PABP class II isomers could be related to an increase in gene expression, the steady-state level of PAB mRNA was investigated. Three-week-old A. thaliana Col-0 plants were mock inoculated or TuMV-infected. At 4, 7 and 17 days post-inoculation (p.i.), leaf tissues were harvested from pools of four plants. qRT-PCR analysis using RNA extracted from TuMV-infected and mock Col-0 at 4, 7 and 17 days p.i. was performed using PAB2-, PAB4- and PAB8-specific primers. With the exception of PAB2 at 4 days p.i., mRNA expression in TuMV-infected plants increased for all class II PAB genes relative to mock inoculated plants (Fig. 1, P>0.05, n=3). Both PAB4 and PAB8 mRNAs showed the largest increases in expression at 17 days p.i., with inductions of 7- and 5.1-fold, respectively. Thus, the observed increase in PAB relative mRNA expression following virus infection correlates with the higher levels of PABP observed in
TuMV-infected *A. thaliana*. It is interesting to note that the mRNA levels (obtained by qRT-PCR) of remaining functional class II *PAB* genes were unchanged in *pab* single or double knockout plants when compared with that of wild-type *A. thaliana* plants. Similarly, *PAB* expression remained unchanged following exposure to mannitol stress (data not shown).

**TuMV RdRp and VPg-Pro interaction with *A. thaliana* PABP2, PABP4 and PABP8 in vitro**

We have previously shown that two TuMV polypeptides, RdRp and VPg-Pro, interact directly with *A. thaliana* PABP2 (Beauchemin & Laliberté, 2007; Dufresne *et al.*, 2008; Léonard *et al.*, 2004). Eight *PAB* genes have been identified in *A. thaliana* (Belostotsky, 2003). The corresponding proteins are divided into four classes based on gene expression and similarity. Whereas class I, III and IV PABP transcripts are produced at low levels and in a tissue-specific manner, class II members (PABP2, PABP4 and PABP8) are highly and broadly expressed and probably constitute the bulk of PABP required for cellular functions. PABP2 and PABP8 are most similar, exhibiting 68% identity at the amino acid level, whereas PABP4 bears 60% identity to PABP2 and 59% identity to PABP8 (see Supplementary Fig. S1, available in JGV Online). This raises the possibility that TuMV VPg-Pro and RdRp could interact with other class II PABPs during infection.

To test for the direct interaction of VPg-Pro and RdRp with PABP2, PABP4 or PABP8, an ELISA-based binding assay was performed. To validate the assay, ELISA plates were coated with metal chelation-purified TuMV 6× histidine-tagged RdRp or VPg-Pro. The coated wells were then incubated with increasing concentrations of purified GST-tagged PABP2. Complex retention was detected using an anti-GST antibody. A typical protein–protein interaction saturation binding curve was observed for both VPg-Pro (Fig. 2a) and RdRp (Fig. 2c). No signal was detected when the wells were coated with a metal-chelating chromatography-purified *E. coli* lysate (data not shown) or when GST-PABP2 was replaced with the GST protein. No synergistic effect on binding was observed when VPg-Pro and RdRp were added simultaneously (data not shown). After validation, we tested the capacity of RdRp and VPg-Pro to bind to PABP class II isoforms. ELISA plates were again coated with recombinant TuMV RdRp or VPg-Pro. The coated wells were then saturated with 25 pmol GST-tagged PABP2, PABP4 or PABP8. For the VPg-Pro-coated plate, the strongest interaction signal was obtained with GST-tagged PABP2 (maximum signal), followed by PABP8 (51% of maximum signal), whereas no signal was detected with PABP4 (Fig. 2d). Data from both experiments were obtained for three biological replicates from a minimum of two technical replicates. The differences recorded were statistically significant (Tukey’s test; *P*<0.05). Overall, this suggests that TuMV RdRp and VPg-Pro interact preferentially with PABP2, but are also capable of interaction with one or both of the other class II PABPs (i.e. PABP4 and PABP8).
Isolation of viable single and double *pab* T-DNA insertion mutants

The collection of *A. thaliana* T-DNA insertional mutants described by Alonso et al. (2003) was screened by PCR for T-DNA disruption of the *PAB2*, *PAB4* and *PAB8* genes. Viable homozygous T₃ knockouts were isolated for all class II *PAB* genes. T-DNA insertions were found in the first exon of the *PAB2* gene, the third exon of the *PAB4* gene and the ninth exon of the *PAB8* gene (Fig. 3a). The corresponding single null mutants were named *pab2*, *pab4* and *pab8*. Viable double mutants were obtained by crossing single *pab* mutants and selfing the F₁ hybrids. The corresponding double mutants were named *pab2 pab4*, *pab4 pab8* and *pab2 pab8*.

The homozygous knockout genotype and the presence of T-DNA insertions were confirmed by PCR on plant genomic DNA (data not shown) and by RT-PCR on expressed mRNA. The *PAB2*, *PAB4* and *PAB8* mRNAs were not detected in the corresponding mutants (Fig. 3b). mRNA levels (obtained by qRT-PCR) of remaining functional class II *PAB* genes were unchanged in *pab* single or double knockout plants when compared with that of wild-type *A. thaliana* plants (data not shown). Immunoblotting using an anti-AtPABP2 polyclonal antibody was also performed on plant protein extracts (Fig. 3c). PABP2 protein was not found in the *pab2* mutants and the PABP4/PABP8 protein band was not detected in *pab4 pab8* mutants. Corresponding *PAB* gene products (mRNA and protein) were not detected in the single and double *pab* mutants, which confirms the knockout genotype.

All attempts to isolate a triple *pab* mutant (by crossing a *pab4 pab8* double mutant with a *pab2* line) failed. To confirm lethality of the *pab2 pab4 pab8* genotype, F₂ progenies of selfed hemizygous double knockout (*pab4 pab8*) lines were sown. Again, a viable *pab2 pab4 pab8* mutant could not be isolated. PCR genotyping of non-germinating seed allowed identification of dead triple knockouts, which indicates that the *pab2 pab4 pab8* genotype is lethal.

TuMV accumulation is reduced in *pab2 pab4* and *pab2 pab8* double A. thaliana knockouts

The interaction of TuMV VPG-Pro and RdRp with PABP suggests that PABP might be a required factor for virus replication and/or for translation of the viral genome. As seen with TuMV and other potyviruses, the disruption of eukaryotic translation initiation factors such as elf4E/elfiso4E and elf4G/elf(iso)4G in *A. thaliana* leads to potyvirus resistance (Duprat et al., 2002; Lellis et al., 2002; Nicaise et al., 2007) which is indicative of their absolute requirement for virus infection.

To test this idea, *pab2*, *pab4* and *pab8* *A. thaliana* single PABP T-DNA knockouts were challenged with TuMV in two independent experiments. Ten plants were inoculated for each genotype. Two weeks p.i., all *pab2*, *pab4* and *pab8* plants showed signs of infection similar to those observed for infected wild-type Col-0 (data not shown). Infection was further confirmed by RT-PCR (data not shown). Thus, none of the individual *PAB* gene products alone was absolutely required for TuMV infection. It is possible that the remaining *PAB* genes can compensate for the loss of the disrupted PABP gene to allow viral replication.

In another set of experiments, TuMV inoculation was repeated on the double mutants *pab2 pab4*, *pab2 pab8* and
*pab4 pab8* to see whether further depletion in PABP cellular level would affect TuMV accumulation. In contrast to *pab* single and *pab4 pab8* mutants, which were usually phenotypically indistinguishable from wild-type plants, *pab2 pab4* and *pab2 pab8* double mutants had growth and development defects (Fig. 4a and Supplementary Fig. S2, available in JGV Online). The triple *pab* knockout genotype (*pab2 pab4 pab8*) was not viable and the effect of complete class II PABP depletion could not be tested. Three week old *pab2 pab4, pab4 pab8, pab2 pab8* and wild-type Col-0 control *A. thaliana* lines were inoculated with TuMV in two independent experiments. All lines showed typical inflorescence stunting that resulted from TuMV infection, indicating that none was completely resistant to TuMV. Despite obvious signs of infection, vegetative tissues from the double mutant line *pab2 pab8* were generally less contorted and stunted, which suggested that they were less susceptible to infection (Fig. 4a).

In order to obtain a quantitative assessment of viral accumulation in these lines, qRT-PCR amplification of the TuMV genomic RNA was performed on TuMV-infected plants at 7 and 17 days p.i. Specifically, total RNA from infected and mock-infected plants was extracted from shoot tissues. An aliquot of this (1 μg) was reversed transcribed to cDNA and RNA concentration was evaluated by qRT-PCR. Relative viral RNA accumulation was calculated; threshold cycle values were obtained for double mutant lines versus wild-type Col-0 plants. The *ACT2* housekeeping gene was used to normalize the data. Statistical analyses of qRT-PCR were conducted using REST MCS relative expression software, which uses a pairwise fixed reallocation randomization test (Pfaffl et al., 2002). All experiments were replicated independently at least twice. Significant reductions in relative TuMV RNA levels were recorded in the *pab2 pab8* line, with reductions of 2- and 3.5-fold at 7 and 17 days p.i., respectively (Fig. 4b). Relative TuMV RNA accumulation in *pab2 pab4* was 1.8-fold less than that of infected wild-type Col-0 line at 17 days p.i. (Fig. 4b). No significant difference was measured for the *pab4 pab8* line. These experiments indicate that partial PABP depletion, in particular that of PABP2, although not sufficient to confer complete resistance, can impede the replication cycle of TuMV in the cell. The decrease in viral RNA accumulation may well be a reflection of a general alteration in protein synthesis or a non-specific consequence of the developmental defects of these plants. However, the phenotype of the different mutants does not always reflect the level of viral RNA accumulation. For example, *pab2 pab4* plants were more affected in their development than *pab2 pab8* plants (see Supplementary Fig. S2) but they accumulated wild-type levels of viral RNA after 7 days, while *pab2 pab8* plants showed a 50 % decrease in viral RNA level for the same period (Fig. 4b). This suggests that the observed reduction in viral RNA accumulation is not an indirect end result of the physiological state of the mutant plants.

**PABP levels in soluble and microsomal fractions correlate with reduced TuMV accumulation**

PABP2 represents approximately two-thirds of the class II PABPs produced in *A. thaliana* (Fig. 3c). Since *pab2 pab4* and *pab2 pab8* lines (but not the *pab4 pab8* mutant) showed reduced viral RNA accumulation, this could be related to a reduction in overall PABP concentration in the cell. However, the possibility exists that the reduced virus accumulation observed might result from a pleiotropic effect of PABP depletion.

We have recently reported redistribution of PABP to membrane-associated fractions in TuMV-infected *B. per*viridis* (Beauchemin & Laliberté, 2007). Through agroinfiltration of fluorescent reporter constructs in *Nicotiana benthamiana*, we also showed that, upon co-expression of the TuMV 6K-VPg-Pro protein, which induces cytoplasmic vesicle formation, PABP2 was found within these

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**Fig. 4.** Infection of PABP double mutants and wild-type Col-0 lines with TuMV. (a) Representative photograph of mock (M) and TuMV-infected (Inf) PABP double mutants (*pab2 pab4, pab4 pab8* and *pab2 pab8*) and wild-type Col-0 line at 17 days p.i. (b) Relative quantification of TuMV genomic RNA in infected PABP double mutants (*pab2 pab4, pab4 pab8* and *pab2 pab8*) and wild-type Col-0 line at 7 and 17 days p.i. by qRT-PCR amplification using primers flanking the coat protein region. Bootstrap analysis performed using REST multicomponent analysis software (10,000 iterations). *, Significantly different from Col-0 reference (*P*<0.05, *n*=3). The dashed line represents the ratio observed in wild-type infected Col-0. The *ACT2* gene was used to normalize the data.
organelles (Beauchemin & Laliberté, 2007; Dufresne et al., 2008). Recruitment of PABP within these virus-induced vesicles derived from endoplasmic reticulum membranes is thought to be important as potyviral replication occurs in association with membranes (Beauchemin et al., 2007; Beauchemin & Laliberté, 2007; Schaad et al., 1997). Therefore, depletion of PABP levels in the membrane fraction would impede the role of PABP in viral replication and result in reduced virus accumulation.

To assess PABP class II isoform levels following TuMV infection in each of the knockout lines, subcellular fractionation experiments were conducted. Leaves of infected plants were harvested 12 days p.i., and soluble (S30) and membrane-enriched (P30) fractions were separated by centrifugation. Proteins from each fraction were separated by SDS-PAGE and subjected to immunoblot analysis with a rabbit serum raised against Arabidopsis AtPABP2. Compared with wild-type TuMV-infected Col-0 plants, overall PABP levels were reduced in all pab double A. thaliana mutants (Fig. 5). The TuMV pab4 pab8 line showed PABP levels that corresponded to approximately 86–88% of what was found in infected Col-0 plants (total, 87.7%; S30, 86.63%; P30, 87.7%). Both pab2 pab4 and pab2 pab8 lines showed a more pronounced decrease in overall PABP levels. In pab2 pab4 plants, the proportion of PABP compared with the wild-type ranged from 18 to 38% (total, 38.4%; S30, 31.4%; P30, 18.1%). These proportions were reduced even further (ranging from 26 to 7%) in pab2 pab8 plants (total, 26.2%; S30, 22.6%; P30, 6.7%). It must be noted that PABP levels were disproportionately reduced in the membrane-associated fraction in both of these mutants. Thus, TuMV accumulation in these lines correlated with cellular PABP concentration in A. thaliana, and more precisely with the concentration of PABP in membranes, which is anticipated to be important for efficient virus infection.

**DISCUSSION**

A. thaliana PABP2 interacts with RdRp and VPg-Pro polypeptides of TuMV (Beauchemin & Laliberté, 2007; Dufresne et al., 2008). These interactions with PABP appear to be important as they localize within virus-induced vesicles, where potyvirus replication reportedly takes place (Beauchemin & Laliberté, 2007; Dufresne et al., 2008). In the present study, we investigated whether PABP was required for potyvirus propagation in A. thaliana. Using a reverse-genetics approach, none of the single PABP knockout plants that were tested were found to be immune to TuMV infection. However, virus multiplication was significantly inhibited in pab2 pab4 and pab2 pab8 double mutants and was well correlated with PABP levels in membranes. These results suggest that a minimal threshold of PABP is required for efficient multiplication of TuMV.

Translation initiation factors in plants are encoded by small gene families. In A. thaliana, four genes [eIF(iso)4E1, eIF4E2, eIF4E3 and eIF(iso)4E] encode proteins of the eIF4E family and three genes [eIF4G1 and eIF(iso)4G1 and eIF(iso)4G2] encode proteins of the eIF4G family (http://research.cm.utexas.edu/kbrowning/flat/). The different isoforms appear to carry out complementary biological functions as demonstrated by the lack of specific phenotypes in plants with disrupted eIF4E/4G isomers (Duprat et al., 2002; Nicaise et al., 2007; Sato et al., 2005). An analogous role for PABP is demonstrated here, as individual deletion of constitutive and highly expressing PABP paralogues (i.e. PAB2, PAB4 and PAB8 genes) has little effect on A. thaliana growth and development.

Despite apparent functional redundancy, the viral requirement for eIF4E/4G or eIF(iso)4E/4G isoforms is not functionally interchangeable (Robaglia & Caranta, 2006). TuMV, for instance, specifically requires eIF(iso)4E or both eIF(iso)4G1 and eIF(iso)4G2 isoforms for successful infection, while CiYVV recruits eIF4E and eIF4G. The only potyvirus known to deviate from this rule is pepper vein mottle virus, which can use both eIF4E and eIF(iso)4E to achieve pepper infection (Ruffel et al., 2006). This selective requirement by most viruses is likely to be a consequence of co-evolution between different plant–virus pairs (Charron et al., 2008). Experimental evidence gathered so far has demonstrated that the resistance phenotype relates to the binding capacity of
VPg for one or the other of the eIF4E isoforms (Khan et al., 2006; Léonard et al., 2000; Yeam et al., 2007). The important structural differences found in the two eIF4F isoforms probably make it difficult to maintain interaction with both isomers.

The present work shows that VPg-Pro and RdRp of TuMV interact not only with PABP2 but also with PABP4 and/or PABP8 isomers in A. thaliana. This would explain why single pab2, pab4 or pab8 knockout plants were susceptible to TuMV infection; the virus could still utilize remaining PABP paralogues that are expressed in the cell. Further depletion of PABP in double knockout plants resulted in reduced TuMV RNA accumulation and confirms that PABP has a role to play in potyvirus infection. The effect appears to be additive, as it correlated with overall cellular PABP levels; PABP2 was relatively more important as it is the most abundant isoform. Our results indicate that A. thaliana triple pab knockouts are not viable. The absence of complete resistance in double knockouts might therefore result from the presence of residual PABP in the cell. We must underline the fact that VPg-Pro interacted with PABP2, PABP4 and PABP8, while RdRp interacted with PABP2 and PABP8 and apparently not with PABP4. The fact that double mutant pab2 pab8 plants were susceptible to TuMV suggests that the RdRp/PABP interaction is not strictly required for virus replication or that RdRp can possibly interact with the tissue-specific and weakly expressed class I, III and IV PABP.

Rather than a reduction in PABP levels through cleavage, as seen with some animal viruses, PABP concentration in TuMV-infected cells was substantially increased. Results obtained here indicate that it can be related to an increase in PAB mRNA steady-state levels. This upregulation of PAB genes is also interesting; we also found that their expression is not influenced by osmotic stress or by PAB gene disruption (data not shown). This suggests that a virus-specific mechanism could be responsible for PABP upregulation, as seen with the upregulation of other genes associated with protein synthesis following TuMV infection (Yang et al., 2007).

The role of PABP in potyvirus infection remains to be clearly defined. An obvious possibility is that PABP, like the canonical translation initiation factors eIF(iso)4E and eIF(iso)4G, is needed for the closed-loop conformation of viral RNA linking VPg and eIF(iso)4G to the viral poly(A) tail in a VPg–eIF(iso)4E–eIF(iso)4G–PABP protein bridge. The latter would be necessary for efficient cap-independent translation of the viral RNA and a potential means of competing with cellular mRNA for the translation machinery. Since these interactions might also sequester key components from cap-dependent target cellular mRNA, they could also simultaneously inhibit cellular translation.

Another hypothesis is that the dual interaction of the RdRp with both PABP and VPg-Pro may serve to switch from viral translational to replication mode. As the genome is translated, RdRp would accumulate and its combined interaction with VPg-Pro (Léonard et al., 2000) and PABP (Dufresne et al., 2008; Wang et al., 2000) bound to viral RNA would evict or modify conformation of the eIF4F complex, dislodge ribosomes engaged on the viral RNA and allow replication to proceed. The latter explanation is appealing, as VPg-Pro can form a tripartite complex with PABP2 and eIF(iso)4E in vitro (Beauchemin & Laliberté, 2007). However, the ability of RdRp to antagonize this tripartite complex remains to be tested.

PABP is an important regulator of mRNA degradation that acts by protecting the 3’ poly(A) tail of mRNAs. This raises the possibility that depletion of PABP cellular levels exposes the viral RNA to increased degradation and would explain the reduced TuMV RNA levels observed in PABP-depleted knockout plants.

Collectively, our data extend previous findings on the importance of PABP in the infectious cycle of TuMV (Beauchemin & Laliberté, 2007; Dufresne et al., 2008). As A. thaliana single and double PABP knockouts are now available, it will be interesting to quantify how other viruses multiply in these mutant backgrounds. Future experiments should aim to carefully dissect whether the requirement for PABP involves viral translation and/or targets RdRp activity. The use of in vitro translation systems and RdRp assays may therefore reveal more precisely the mechanistic function of PABP in the potyvirus life cycle.

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