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RBP45 and RBP47, two oligouridylate-specific hnRNP-like proteins interacting with poly(A)$^+$ RNA in nuclei of plant cells

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ABSTRACT

Introns in plant nuclear pre-mRNAs are highly enriched in U or U + A residues and this property is essential for efficient splicing. Moreover, 3′-untranslated regions (3′-UTRs) in plant pre-mRNAs are generally UA-rich and contain sequences that are important for the polyadenylation reaction. Here, we characterize two structurally related RNA-binding proteins (RBPs) from *Nicotiana plumbaginifolia*, referred to as RBP45 and RBP47, having specificity for oligouridylates. Both proteins contain three RBD-type RNA-binding domains and a glutamine-rich N-terminus, and share similarity with Nam8p, a protein associated with U1 snRNP in the yeast *Saccharomyces cerevisiae*. Deletion analysis of RBP45 and RBP47 indicated that the presence of at least two RBD are required for interaction with RNA and that domains other than RBD do not significantly contribute to binding. mRNAs for RBP45 and RBP47 and mRNAs encoding six related proteins in *Arabidopsis thaliana* are constitutively expressed in different plant organs. Indirect immunofluorescence and fractionation of cell extracts showed that RBP45 and RBP47 are localized in the nucleus. In vivo UV crosslinking experiments demonstrated their association with the nuclear poly(A)$^+$ RNA. In contrast to UBP1, another oligouridylate-binding nuclear three-RBD protein of *N. plumbaginifolia* (Lambermon et al., *EMBO J*, 2000, *19*:1638–1649), RBP45 and RBP47 do not stimulate mRNA splicing and accumulation when transiently overexpressed in protoplasts. Properties of RBP45 and RBP47 suggest they represent hnRNP-proteins participating in still undefined steps of pre-mRNA maturation in plant cell nuclei.

Keywords: 3′-UTR; *Arabidopsis*; RBD; RNA-binding proteins; RNA processing; UA-rich sequences

INTRODUCTION

mRNAs in eukaryotes are synthesized as precursors that undergo a series of elaborate processing events in the nucleus. Splicing and polyadenylation are particularly complex reactions. They depend on numerous *cis*-acting elements in pre-mRNA that are recognized by different protein and ribonucleoprotein (RNP) factors (reviewed by Krämer, 1996; Colgan & Manley, 1997; Wahle & Ruegsegger, 1999). During splicing, sequences contributing to intron recognition and splice site selection include universally important elements such as appropriate 5′ and 3′ splice sites (5′ss and 3′ss), the branch site, and other signals that are either organism or pre-mRNA specific. Examples of these other signals include 3′ss proximal polypyrimidine tracts that function primarily in metazoa, and splicing enhancers and regulators, which are usually essential for splicing of a particular mRNA or group of mRNAs (reviewed by Krämer, 1996; Lopez, 1998; Blencowe, 2000). Selection of the polyadenylation sites in pre-mRNAs also depends on a number of *cis* elements. In vertebrates, they include a highly conserved sequence, AAUAAA, located 20–30 nt upstream of the cleavage/polyadenylation site, and the DSE elements that are U- or GU-rich and are usually located downstream of the cleavage site (reviewed by Colgan & Manley, 1997; Keller & Minvielle-Sebastia, 1997; Wahle & Ruegsegger, 1999).

Pre-mRNA splicing is mediated by the spliceosome, an RNP complex assembled in a stepwise manner from small nuclear RNPs (U-snRNPs) and numerous proteins. U-snRNPs recognize pre-mRNA signals by base pairing, whereas many protein factors (e.g., U2AF or SR proteins) specifically interact with approximately 10-nt-long signals of specific sequence or base composition in pre-mRNA (reviewed by Krämer, 1996; Valcárcel & Green, 1996; Lopez, 1998; Tacke & Manley, 1999;

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Blencowe, 2000). The cleavage/polyadenylation signals are likewise recognized by specific proteins, subunits of the large 3′-end processing complex (reviewed by Colgan & Manley, 1997; Keller & Minvielle-Sebastia, 1997; Wahle & Ruegsegger, 1999). In addition to the components of splicing and polyadenylation machineries, pre-mRNAs in the nucleus interact with tens of RNA-binding proteins, collectively referred to as hnRNP proteins, extensively characterized in mammals and *Drosophila melanogaster* (reviewed by Swanson, 1995; Weighardt et al., 1996; Siomi & Dreyfuss, 1997). Originally, hnRNP proteins were believed to be responsible only for packaging and proper folding of processing substrates, but recently, many have been identified as factors involved in RNA processing, transport, or stability, frequently in the regulation of these processes (reviewed by Siomi & Dreyfuss, 1997; Krecic & Swanson, 1999). The same protein can regulate mRNA maturation at more than one level. An example is hnRNP A1, which is involved in control of splicing and mRNA export (reviewed by Krecic & Swanson, 1999). Very few hnRNP-like proteins have been identified in yeast, but they also emerge as factors contributing to pre-mRNA processing (Wilson et al., 1994; Kessler et al., 1997; Minvielle-Sebastia et al., 1998; reviewed by Swanson, 1995; Krecic & Swanson, 1999).

The basic mechanisms of pre-mRNA processing in plants resemble those in other eukaryotes, although details of this process are poorly characterized due to the lack of appropriate in vitro systems. As in other organisms, mRNA splicing in plants is a two-step reaction, involving a lariat formation. U-snRNPs, many splicing factors, and branch points and splice sites generally are similar to their vertebrate counterparts (reviewed by Simpson & Filipowicz, 1996; Brown & Simpson, 1998; Lorković et al., 2000). Likewise, limited experimentation and inspection of sequence databases indicate that many factors, participating in the polyadenylation reaction in metazoa, are highly conserved in plants (reviewed by Rothnie, 1996). However, despite these similarities, some aspects of splicing and polyadenylation reactions in plants differ significantly from the processes in other organisms. This is best illustrated by the fact that mammalian pre-mRNAs are generally neither accurately nor efficiently processed in plant cells (reviewed by Simpson & Filipowicz, 1996; Brown & Simpson, 1998; Lorković et al., 2000).

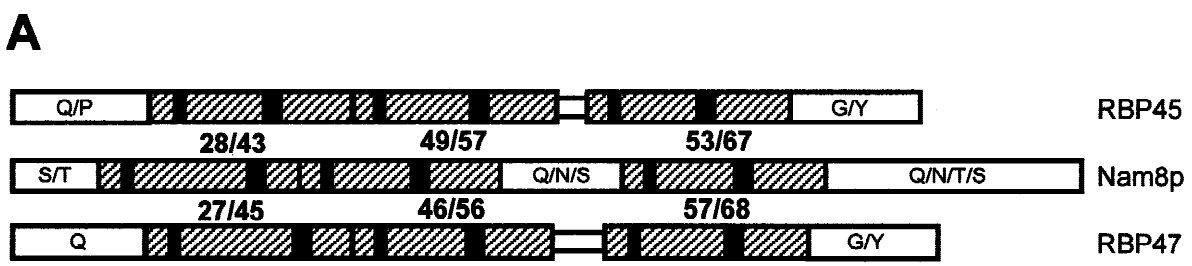
The reasons underlying the specificity of plant splicing have been a subject of intensive studies. A characteristic feature of plant introns is their UA or U richness, as compared to the flanking exons. Previous work has shown that UA- or U-rich elements, usually distributed throughout the introns, greatly increase splicing efficiency in both dicots and monocots (Goodall & Filipowicz, 1989; Luehrsen & Walbot, 1994; Gniadkowski et al., 1996; Ko et al., 1998), and also play an important role in defining intron borders (Lou et al., 1993; McCullough et al., 1993; Luehrsen & Walbot, 1994; Egoavil et al., 1997; reviewed by Schuler, 1998). Analysis of synthetic and natural introns indicated that Us rather than As are functional residues of the elements and that they activate splicing irrespective of their location in the intron (Goodall & Filipowicz, 1989; McCullough et al., 1993; Gniadkowski et al., 1996; Egoavil et al., 1997). Hence, these elements function differently from the metazoan polypyrimidine tracts, which are generally located downstream of the branch point (reviewed by Krämer, 1996). Notably, 3′-untranslated regions (3′-UTRs) in plant mRNAs are also generally UA-rich (Luehrsen & Walbot, 1994; reviewed by Rothnie, 1996) and the upstream elements enhancing utilization of the cauliflower mosaic virus polyadenylation site have a consensus UUUGUA (reviewed by Rothnie, 1996).

To gain more insight into the function of U-rich elements in plant mRNA processing, we are characterizing proteins interacting with nuclear pre-mRNAs in plants. We have recently described a *Nicotiana plumbaginifolia* nuclear RNA-binding protein, UBP1, with specificity for oligouridylates. UBP1 interacts with plant UA-rich introns and 3′-UTRs in vitro, and with nuclear poly(A)$^+$ RNA in vivo. Transfection experiments have revealed two apparently independent activities of UBP1: stimulation of splicing of suboptimal introns and an increase in the accumulation of reporter mRNAs that contain inefficiently spliced introns or are intronless. The latter effect appears to be due to UBP1 interacting with the 3′-UTR and protecting mRNA from exonucleolytic degradation (Lambermon et al., 2000). We have also characterized plant homologs of the splicing factor U2AF that binds to 3′ss-proximal polypyrimidine tracts and helps to position U2 snRNP at the branch point in metazoa. Recombinant forms of the two large-subunit isoforms of the plant U2AF have specificity for oligouridylates and functionally substitute for the human U2AF (Domon et al., 1998). By analogy with metazoan splicing (reviewed by Krämer, 1996), it is likely that U2AF binds to the 3′ss-proximal U-rich region of plant introns. In this work, we describe the cloning and characterization of two additional RNA-binding proteins, which have specificity for oligouridylates and interact with poly(A)$^+$ RNA in plant cell nuclei.

RESULTS

cDNAs and genes encoding RNA-binding proteins RBP45 and RBP47 in *N. plumbaginifolia* and *A. thaliana*

Using the PCR approach, we have previously characterized cDNA sequences from *Nicotiana tabacum* encoding 15 distinct RBD-type RNA-binding proteins



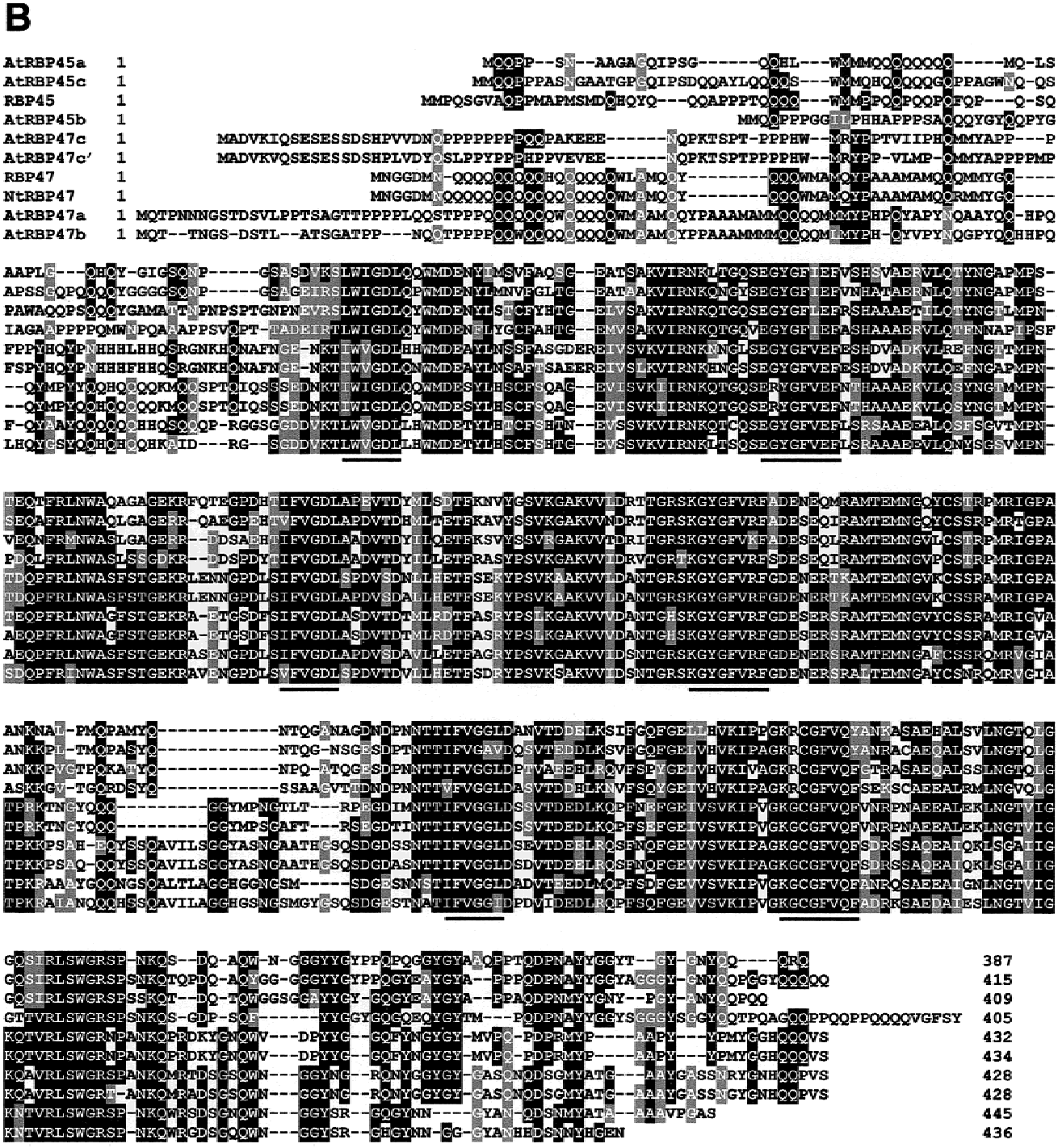


FIGURE 1. *See caption on facing page.*

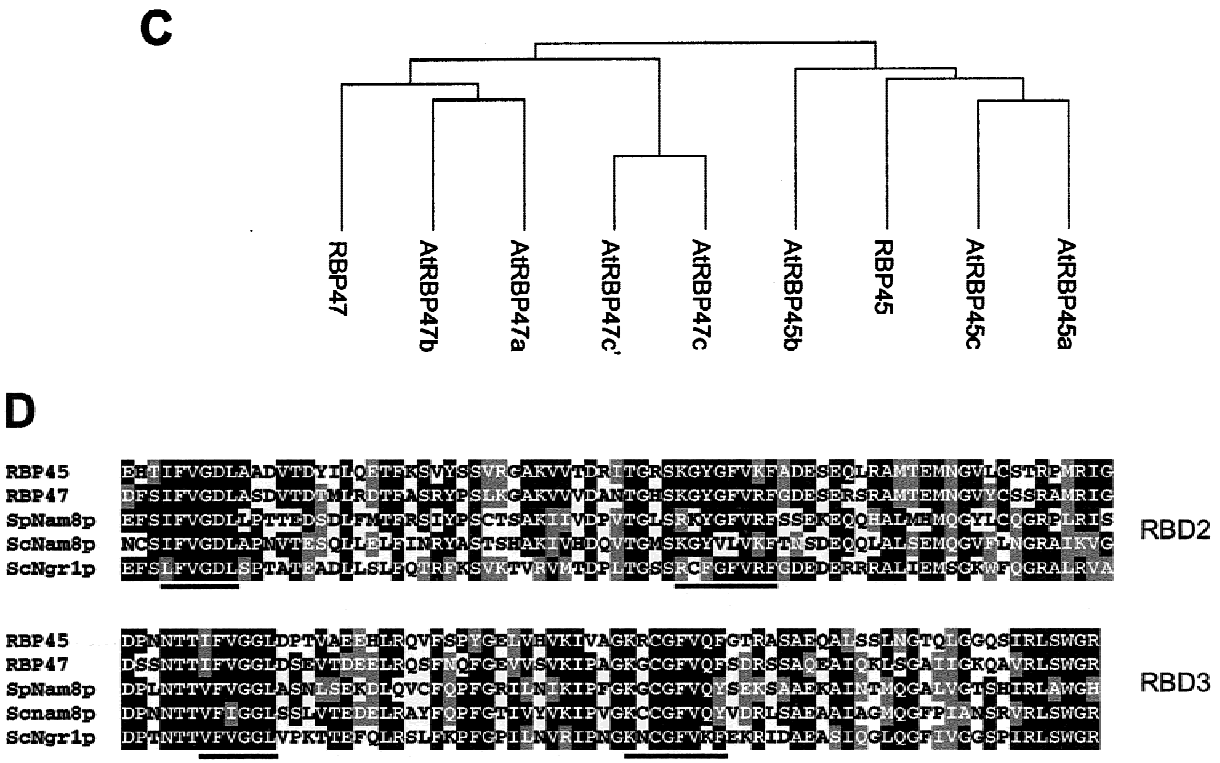


FIGURE 1. (*Figure continued from facing page.*) Sequence analyses of RBP45 and RBP47. **A**: Schematic representation of RBP45 and RBP47 compared with the yeast Nam8p. RBD domains are shown as hatched boxes with the RNP1 and RNP2 consensus sequences highlighted in black. Domains enriched in specific indicated amino acids are shown as open boxes. Narrow bars indicate regions without any obvious characteristic features. Percent identity and similarity between respective RBD domains is indicated. **B**: Sequence alignment of RBP45 and RBP47 with related proteins from *N. tabacum* (Nt) and *A. thaliana* (At). Sequences of AtRBP45 and AtRBP47 proteins were deduced from the ESTs and genomic sequences (GenBank accession numbers AB005232, AC007296, AL035440, AC007519, AP000419, and AC011807). The sequence of NtRBP47 is from Seguin et al. (1997), except for the corrected amino acid sequence QQRMMYGQQYMPY at positions 48–59 (GenBank accession number U90212; our unpubl. results). **C**: Dendrogram of RBP45 and RBP47 proteins from *N. plumbaginifolia* and *A. thaliana*. The dendrogram and sequence alignments were generated with the PileUp program, using default parameters (Genetics Computer Group, Madison, Wisconsin). **D**: Alignments of the second and third RBDs of RBP45 and RBP47 with respective RBDs of the *S. cerevisiae* (Sc) Nam8p (Q00539) and Ngr1p (P32831) proteins and the *S. pombe* (Sp) Nam8p-like protein (Z99292). Conserved amino acids in **B** and **D** were shaded using the BOXSHADE program available at http://www.isrec.isb-sib.ch/software/BOX_form.html. Amino acids identical or similar in more than 50% of analyzed sequences have a black and gray background, respectively. RNP2 (6 amino acids) and RNP1 (8 amino acids) motifs of RBD domains are underlined.

(Mieszczak et al., 1992). These cDNA fragments were then used as hybridization probes to screen the *N. plumbaginifolia* cDNA library. Among the characterized clones we have identified two cDNAs encoding RNA-binding proteins named RBP45 and RBP47. Schematic structures of the proteins are shown in Figure 1A. Each protein contains three RBDs, comprising the RNP1 and RNP2 consensus sequences, a glutamine-rich domain at the N terminus, and a C-terminal domain enriched in glycines and tyrosines. In each protein, the first two RBDs are adjacent to each other, and the third one is separated from them by a spacer of variable length. RBP45 and RBP47 are 55% identical and 59% similar and may represent members of the same protein family (see below).

Database searches indicated that multiple variants of RBP45 and RBP47 are encoded in plant genomes. In *A. thaliana*, mRNAs encoding at least three RBP45-like and four RBP47-like proteins are expressed, as revealed by sequences of the genes derived within the *A. thaliana* genome project and the available EST clones, sequences of which have been completed by us. Likewise, multiple protein variants are expressed in rice (our unpubl. observations). The alignment of *N. plumbaginifolia* and *A. thaliana* proteins is shown in Figure 1B. It also includes a protein very similar to RBP47, identified recently in *N. tabacum* as a transcription factor interacting with double-stranded DNA (Seguin et al., 1997; see Discussion). RBP45 and RBP47 proteins represent two subgroups of the larger protein family (Fig. 1C). This is also supported by the organization of the *A. thaliana* genes (for GenBank accession numbers, see legend to Fig. 1): they all contain five introns, present at identical positions. Members of the AtRBP45 and AtRBP47 subgroups are 62–79% and 56–80% identical, respectively; most of the sequence differences

are found in terminal auxiliary domains, and in the linker regions between RBD2 and RBD3. It should be noted that the N-terminal domain in AtRBP47c and AtRBP47c′ proteins is proline/histidine rich rather than glutamine rich. AtRBP47c and c′ are 92% identical. Their genes are adjacent to each other, suggesting a recent gene duplication event.

Comparison of RBP45 and RBP47 with proteins from nonplant organisms revealed sequence similarity (27–28, 46–49, and 53–57% identity in RBD1, RBD2, and RBD3 domains, respectively) with the three-RBD protein Nam8p of *Saccharomyces cerevisiae* (Fig. 1A). Nam8p has recently been demonstrated to be a part of the U1 snRNP and to play a role in splicing of suboptimal introns in yeast (Gottschalk et al., 1998; Puig et al., 1999; see Discussion). The alignment of RBD2 and RBD3 domains of the *N. plumbaginifolia* RBP45 and RBP47 with respective domains of Nam8p and two other Nam8p-like proteins, the *S. cerevisiae* Ngr1p and the *Schizosaccharomyces pombe* Nam8p, is shown in Figure 1D.

RBP45 and *RBP47* are constitutively expressed

Northern analysis indicated that RBP45 and RBP47 are encoded by mRNAs of approximately 1.7 and 1.8 kb, respectively. Similar mRNA levels were found in leaves, roots, and stems of *N. plumbaginifolia* plants. No differences were observed in mRNA levels in leaves of plants grown in periodic light or kept in the dark for either 24 or 48 h (data not shown).

Using mRNA-specific complementary probes and RNase A/T1 mapping procedure we have determined levels of expression of six different *A. thaliana RBP45/47* genes in different plant organs. The investigated genes appear to be expressed in leaves, stems, and flowers as well as in seedlings and the cell suspension culture. Some differences in the expression levels are evident. For example, *AtRBP45a* appears to be more strongly expressed in flowers and cells grown is suspension culture. In addition, expression of *AtRBP47b* is lower than that of other genes in all plant organs (Fig. 2).

Generation of antibodies specific for RBP45 and RBP47

We have raised mouse monoclonal antibody against RBP47 (mAb RB4), and rabbit polyclonal antibody against the C-terminal peptide of RBP45 (Ab α-45). Specificity of antibodies was tested by immunoblot analysis of protoplast lysates prepared from either *N. plumbaginifolia* mesophyll or suspension culture cells, or from the *N. tabacum* BY-2 cell suspension culture. The anti-RBP47 mAb RB4 visualized a single protein band with an electrophoretic mobility of approximately 53 kDa. The polyclonal Ab α-45 recognized a major protein band of apparent molecular mass of 52 kDa and a less intense band of slower mobility (Fig. 3A). Additional western blots, performed with purified recombinant RBP45 and RBP47 proteins, demonstrated that each antibody is specific for the protein used as the immunization antigen (data not shown).

Subcellular localization of RBP45 and RBP47

Specific antibodies and the tagging approach were used to analyze subcellular localization of RBP45 and RBP47 proteins. Endogenous proteins were localized in BY-2 cells by indirect immunofluorescence, using specific primary antibodies and either anti-mouse Cy3-labeled (Fig. 3B, RBP47) or anti-rabbit FITC-labeled (Fig. 3B; RBP45) secondary antibodies. Images were analyzed with confocal laser scanning microscopy. Both proteins localized to the nucleus, with no or very little cytoplasmic staining. In nuclei, they showed a diffuse distribution throughout the nucleoplasm with the nucleoli excluded. No labeling of defined nuclear structures, for example, coiled bodies, was apparent [compare with the U2B″ panel, showing staining with mAb 4G3, specific for the U2 snRNP protein U2B″, which is present in the nucleoplasm and coiled bodies (Beven et al., 1995)]. Similar nuclear localization was observed when the influenza hemaglutinin (HA)-epitope-tagged RBP45 and RBP47 were expressed in transfected *N. plumbaginifolia* mesophyll protoplasts, and proteins visualized with a rat anti-HA primary mAb and anti-rat FITC-coupled secondary antibody (Fig. 3B, panels RBP45-HA and RBP47-HA).

The intracellular distribution of the proteins was also studied by cellular fractionation. Lysates prepared from *N. plumbaginifolia* mesophyll protoplasts were separated into nuclear and cytoplasmic fractions, and the distribution of endogenous RBP45 and RBP47 determined by western blots (Fig. 3C). Analysis of the distribution of the nuclear U2B″ protein indicated that there is no appreciable contamination of the cytoplasmic fraction with nuclear proteins (Fig. 3C). The cytosolic isoform of cysteine synthase was found exclusively in the cytoplasmic fractions, as determined by western analysis with an antibody raised against the *A. thaliana* enzyme (data not shown). Western analysis of the *N. plumbaginifolia* fractions with antibodies against RBP45 and RBP47 indicated that only a small fraction, at most 5%, of each protein is recovered in the cytoplasmic material (Fig. 3C).

To distinguish whether low signals seen in cytoplasmic fractions of *N. plumbaginifolia* cells originate from investigated proteins or from other RBP45- and RBP47-like proteins having similar electrophoretic mobilities, we determined cellular distribution of both proteins expressed in protoplasts as HA epitope fusions. Analysis of nuclear and cytoplasmic fractions with anti-HA mAbs demonstrated that they are detectable only in the nuclear material (Fig. 3D).

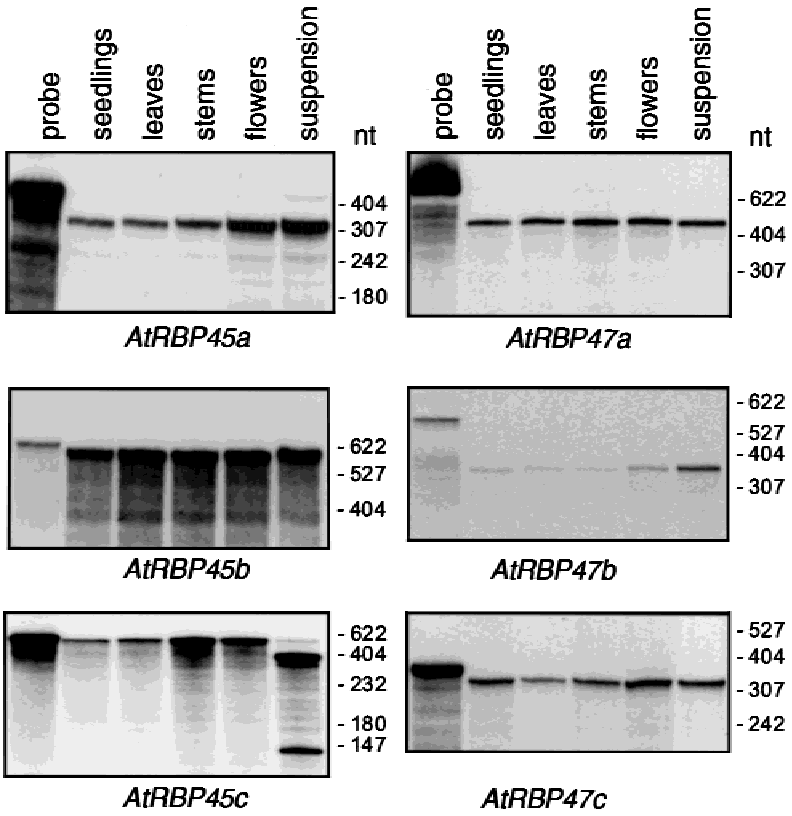


FIGURE 2. Expression analysis of *Arabidopsis RBP45* and *RBP47* genes by RNaseA/T1 mapping. Mappings were performed with 5 μg of total RNA isolated from seedlings, leaves, stems, flowers (Columbia ecotype), and cell suspension (Landsberg *erecta* ecotype). Two bands observed with *AtRBP45c* result from the polymorphism between the two ecotypes. Size markers in nucleotides (nt) are given on the right side of each panel.

RBP45 and RBP47 are associated with poly(A)$^+$ RNA in vivo

We investigated whether RBP45 and RBP47 interact with poly(A)$^+$ RNA in vivo. Protoplasts prepared from *N. plumbaginifolia* cells grown in suspension were irradiated with UV, and extracts prepared from the isolated nuclei were fractionated on oligo(dT)-cellulose columns in the presence of SDS. Western analysis of proteins coeluting with poly(A)$^+$ RNA indicated that RBP45 and RBP47 are associated with RNA isolated from UV-irradiated but not control protoplasts (Fig. 4). Likewise, RZ-1, which has been characterized previously as a protein associated with 60S RNP complex in extracts from tobacco nuclei (Hanano et al., 1996), was found to associate with the nuclear poly(A)$^+$ RNA in intact protoplasts. In the western analysis, we also included mAb against the U2-snRNA-associated protein U2B″. U2B″ was not detected in fractions containing nuclear poly(A)$^+$ RNA from UV-irradiated protoplasts, indicating that the crosslinking procedure specifically identifies proteins interacting directly with poly(A)$^+$ RNA.

In summary, from the immunofluorescence and cell fractionation studies, and the in vivo UV-crosslinking experiments, we conclude that RBP45 and RBP47 are nuclear proteins associating with the poly(A)$^+$ RNA.

RNA-binding specificity of RBP45 and RBP47

RBP45 and RBP47 were overexpressed in *Escherichia coli* as GST fusions and purified. Their RNA-binding properties were determined by UV crosslinking. Both proteins were found to interact with ^{32}P-labeled AU-rich RNA fragments representing either intron 3 of the *A. thaliana* Rubisco activase gene (*rca*; Fig. 5A) or a synthetic intron Syn7 (Goodall & Filipowicz, 1989; data not shown). To establish the nucleotide specificity of binding, four different ribohomopolymers were tested as competitors in the binding assay with labeled *rca* RNA. For both proteins, poly(U) was the most efficient competitor, already having a strong effect at 25-fold molar excess. Some competition with either of the two proteins was also reproducibly observed in the presence of 100-fold excess of poly(G), whereas poly(A) and poly(C) did not compete with *rca* RNA binding even when present at 400-fold excess (Fig. 5A).

To assess contribution of individual RBDs and/or accessory terminal domains to RNA binding, deletion analyses of RBP45 and RBP47 were performed. Schematic structure of all investigated deletion mutants of the GST-RBP45 fusion is shown in Figure 5B, and the data on their purity and UV crosslinking to *rca* RNA are presented in Figures 5C and 5D, respectively. Apparent K_d

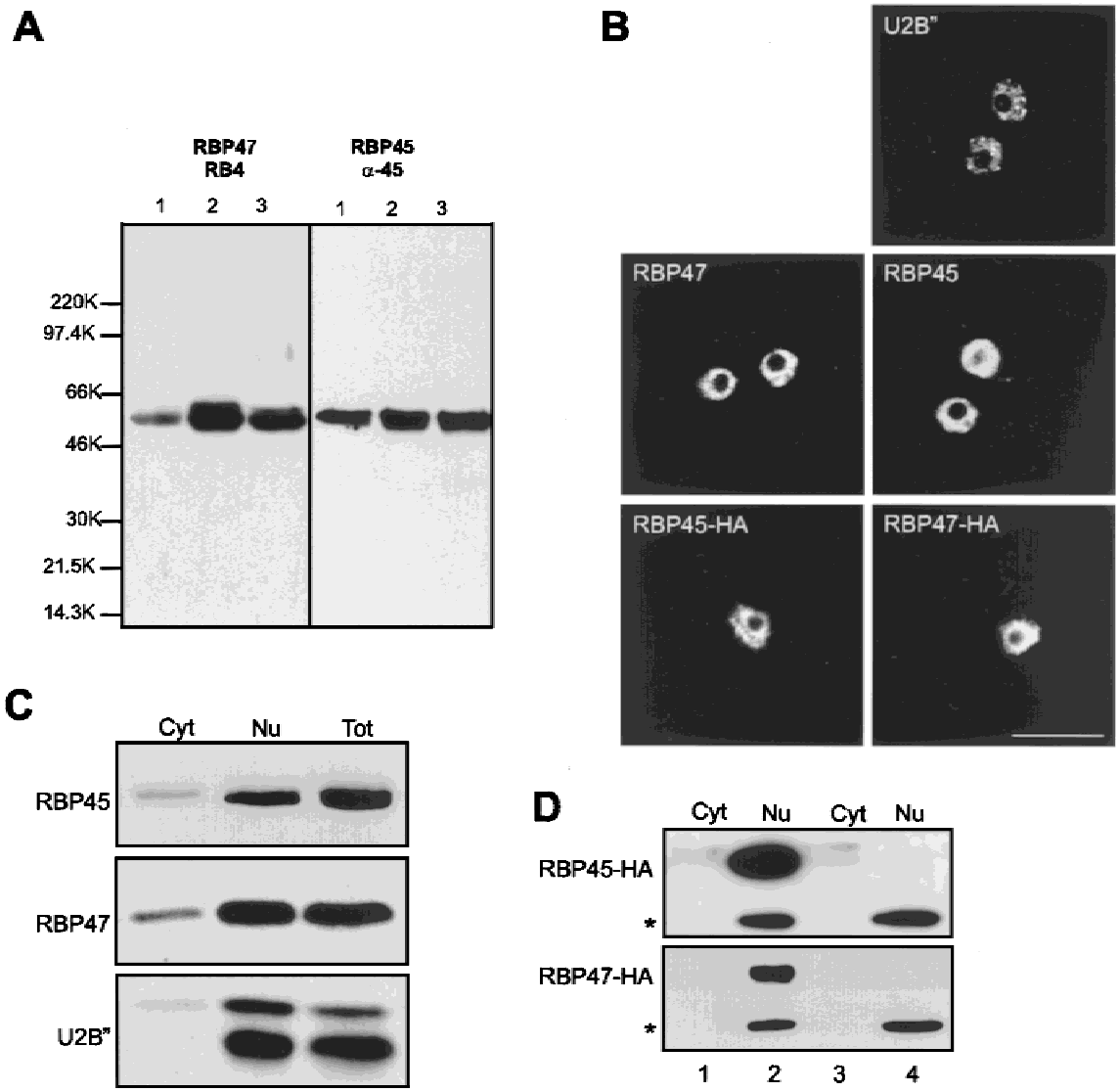


FIGURE 3. Cellular localization of RBP45 and RBP47 proteins. **A**: Specificity of antibodies raised against *N. plumbaginifolia* proteins. Total cell extracts were prepared from *N. plumbaginifolia* mesophyll (lanes 1) or cell suspension (lanes 2) protoplasts, and *N. tabacum* BY-2 cell suspension protoplasts (lanes 3), run on 10% SDS-PAGE and analyzed by western blotting. Antibodies used for western analysis and the proteins to which they were raised are indicated at the top. Protein size (kDa) markers are indicated. **B**: Cellular localization of RBP45 and RBP47 proteins studied by indirect immunofluorescence. Localization of endogenous RBP45 (polyclonal antibody α-45), RBP47 (mAb RB4), and nuclear U2B″ protein (mAb 4G3) in tobacco BY-2 cells (three upper panels). Localization of HA-epitope-tagged RBP45 and RBP47 proteins in transfected *N. plumbaginifolia* protoplasts detected with rat α-HA mAb 3F10 (two bottom panels). No significant staining of BY-2 cells or *N. plumbaginifolia* protoplasts was detected when primary antibodies were omitted. Likewise, no staining of untransfected protoplasts was seen (not shown). Bar = 20 μm. **C**: Subcellular localization of RBP45 and RBP47 studied by fractionation of nontransfected *N. plumbaginifolia* mesophyll protoplasts. Western blots were analyzed using mAb RB4 (RBP47), mAb 4G3 (U2B″), and polyclonal antibody α-45 (RBP45). The upper band on the U2B″ panel cross-reacts unspecifically with mouse α-U2B″ mAb. **D**: Fractionation of *N. plumbaginifolia* mesophyll protoplasts transfected with pRBP45-HA (lanes 1 and 2; upper panel) and pRBP47-HA (lanes 1 and 2; lower panel) and with the empty HA-tag vector (pHAT0; lanes 3 and 4; upper and lower panels). Western blots were analyzed with mouse α-HA mAb 12CA5. A nuclear protein marked with an asterisk and cytoplasmic proteins of 50–55 kDa present in *N. plumbaginifolia* extracts cross-react unspecifically with mouse α-HA mAb 12CA5T. Tot: total cell extract; Cyt: cytoplasmic fraction; Nu: nuclear fraction.

values, estimated by the nitrocellulose filter binding assays, and information about specificity of binding, based on the results of competition experiments with poly(U) and poly(G), are summarized in Figure 5B. Deletion of any single RBD domain (mutants RBD1/2, RBD2/3, and RBD1/3) or both terminal accessory domains (mutant RBD1/2/3) did not strongly influence the ability of the protein to bind RNA. Some differences in the apparent K_d values were however observed, and the binding of the protein having RBD3 and C-terminal domains (mutant RBD1/2) deleted was not any more competed by poly(G). Because deletion of the C-terminal, as well

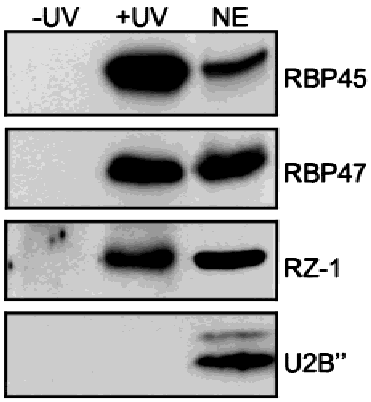


FIGURE 4. In vivo UV-induced crosslinking of RBP45 and RBP47 to poly(A)$^+$ RNA. Eluates from oligo-(dT)-cellulose, obtained from nuclear extracts originating from UV-irradiated (+UV) and control (−UV) protoplasts were analyzed by western blotting using protein-specific antibodies. RBP45 and RBP47 and also RZ-1 proteins coelute with poly(A)$^+$ RNA after crosslinking, whereas the U2B″, a structural component of the U2 snRNP does not. NE: aliquots of nuclear extract prior to oligo(dT)-cellulose fractionation.

as N-terminal, auxiliary domain has no effect on the specificity of binding (mutant RBD1/2/3), it is likely that RBD3 is responsible for the poly(G)-sensitive interaction with RNA. RBD2 alone (mutant RBD2) was able to bind RNA, although with much higher K_d (Fig. 5B,D). Simultaneous deletion of two RBDs, either RBDs 1 and 2 (mutant RBD3) or RBDs 2 and 3 (mutant RBD1), abolished RNA binding nearly completely (mutant RBD3 bound about 10% of input *rca* RNA at approximately 10 μM concentration of the protein; see legend to Fig. 5B). Essentially the same results were obtained with deletion mutants of RBP47 (data not shown).

In summary, these results indicate that combination of any two RBDs is sufficient for strong and oligouridylate-specific interaction with RNA, whereas single RBDs either do not bind RNA (RBD1) or bind it very inefficiently (RBD2 and RBD3). The domain RBD3, in combination with either RBD 1 or 2, is required for the poly(G)-specific interaction.

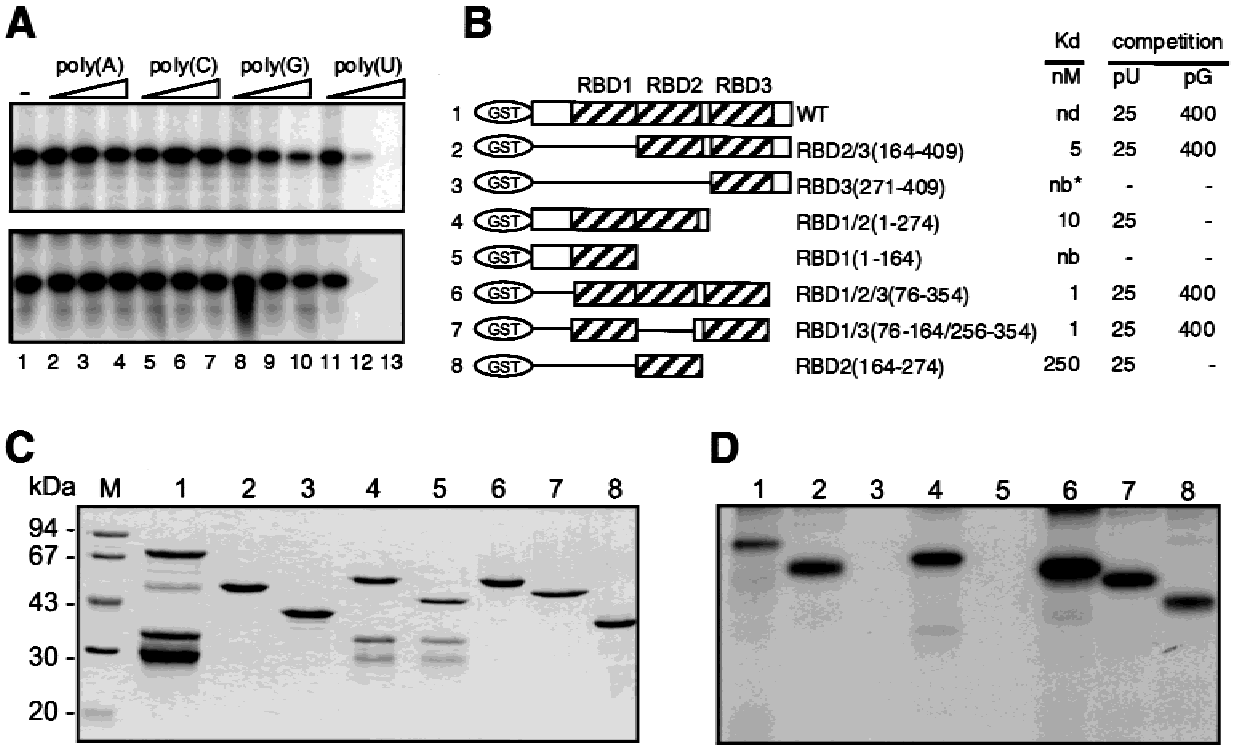


FIGURE 5. Determination of RNA binding specificity of RBP45 and RBP47 and RBP45 deletion mutants. **A**: Nucleotide binding specificity of RBP45 (upper panel) and RBP47 (lower panel) measured by the UV crosslinking/homoribopolymer competition assay using ^{32}P-labeled *rca* RNA. Different indicated polymers were added at 5-fold (lanes 2, 5, 8, and 11), 25-fold (lanes 3, 6, 9, and 12), and 100-fold (4, 7, 10, and 13) excess (calculated in moles of nucleotides) over *rca* RNA (−: crosslinking reactions with no competitor added). Only relevant gel fragments are shown. **B**: Structure of the RBP45 deletion mutants and summary of their nucleotide binding specificity and K_ds. The numbers indicated at each deletion mutant correspond to the first and the last amino acid included. K_d values were measured by the filter binding assay. nd: not determined (due to the high content of incomplete fusion protein; see **C**, lane 1); nb: no binding (nb*, the RBD3 mutant bound only ~10% of the input RNA at 10 μM concentration, making the determination of K_d difficult). The numbers shown in the competition columns represent molar excess of poly(U) (pU) and poly(G) (pG) required for approximately 90% and 50% of competition, respectively. (−): no binding or competition observed. **C:** Coomassie blue-stained gel of RBP45 and its deletion mutants overexpressed and purified on glutathione Sepharose 4B. **D**: UV crosslinking of ^{32}P-labeled *rca* RNA to RBP45 and its deletion mutants. Proteins were analyzed by SDS-PAGE. Lane numbering in **C** and **D** corresponds to the numbering of deletion mutants in **B**.

Comparison of effects of RBP45, RBP47, and UBP1 on splicing and accumulation of reporter RNAs

We have recently characterized the *N. plumbaginifolia* nuclear RNA-binding protein UBP1 that can be cross-linked to U-rich introns and 3′-UTRs in vitro, and that associates with the nuclear poly(A)$^+$ RNA in vivo. When expressed transiently in protoplasts, UBP1 enhances splicing of otherwise inefficiently processed introns and increases the accumulation of reporter mRNAs that contain suboptimal introns or are intronless; the latter effect is likely due to the protein binding to the 3′-UTR and protecting mRNA against exonucleolytic degradation (Lambermon et al., 2000). Many biochemical properties of RBP45 and RBP47 are very similar to those of UBP1. All proteins contain three similarly spaced RBD domains and a glutamine-rich N-terminus, they cross-link to the nuclear poly(A)$^+$ RNA in vivo, and have similar RNA binding specificities.

We have investigated whether overexpression of RBP45 and RBP47 in protoplasts of *N. plumbaginifolia* has effects similar to those identified for UBP1. The SynGC/ClaU reporter, which contains a suboptimal intron with the short 5′ss-proximal U-rich sequence (Gniadkowski et al., 1996; Fig. 6A) was used to assess splicing efficiency. The effect on RNA accumulation was measured with synthetic intronless reporter Syn3 (Lambermon et al., 2000; Fig. 6B). As shown in Figure 6, no stimulation of splicing or accumulation of the investigated reporter RNAs was observed for either RBP45 or RBP47; in fact, their overexpression slightly decreased the yield of the reporter Syn3 RNA. This contrasts with UBP1, expression of which stimulated both RNA splicing and accumulation (Fig. 6A,B). We have verified by western analysis that the HA-epitope-tagged RBP45, RBP47, and UBP1 are expressed in protoplasts at comparable levels (data not shown).

RBD2 and RBD3 domains of RBP45 and RBP47 have considerable sequence similarity with respective RBDs of the yeast protein Nam8p (see above). Nam8p, an integral U1 snRNP protein in yeast, has recently been shown to interact with the intronic 5′ss-proximal U-rich sequence and to stimulate splicing at the adjacent suboptimal 5′ss (Puig et al., 1999). We have tested whether overexpression of either RBP45 or RBP47 could rescue exon skipping in the *A. thaliana apetala* gene that is caused by the A → U mutation at the −2 position in the 5′ss of intron five (*ap3-1*; Jack et al., 1992). This mutation weakens the base-pairing potential of the U1 snRNA with the 5′ss (Fig. 6C), which makes this 5′ss comparable to the 5′ss sites in yeast introns targeted by Nam8p (Puig et al., 1999). Importantly, the region positioned downstream of the *ap3-1* intron 5′ss is enriched in U residues. As shown in Figure 6C, neither RBP45 nor RBP47 stimulated utilization of the mutant splice site of the *ap3-1* transcript.

Several other approaches (see Discussion) were also used to establish the function of RBP45 and RBP47 proteins. To date, they did not provide conclusive answer as to the biological role(s) of the proteins in mRNA maturation.

DISCUSSION

In this work, we have characterized two *N. plumbaginifolia* nuclear RNA-binding proteins, RBP45 and RBP47. The two proteins, and also their counterparts in *A. thaliana*, contain three RBD domains of which the two N-proximal domains are adjacent to each other and are separated from third one by a linker of variable length. In their domain organization, the plant proteins resemble several families of three-RBD proteins identified in metazoa and yeast. These families include metazoan ELAV-like proteins [e.g., *Drosophila elav*, mammalian HuC, HuD, HuR, and Hel-N1 (Robinow et al., 1988; Szabo et al., 1991; Kim & Baker, 1993; King et al., 1994; Good, 1995; Ma et al., 1996; Antic & Keene, 1997)], metazoan TIA-1 and TIAR (Beck et al., 1996; Dember et al., 1996), yeast Nam8p (Gottschalk et al., 1998) and Pub1p (Anderson et al., 1993; Matunis et al., 1993), some vertebrate hnRNP proteins [e.g., hnRNP proteins F and H (Gamberi et al., 1997, and references therein)], and also plant protein UBP1 (Lambermon et al., 2000). Although the exact function of only a few of these proteins is known, most are implicated in the regulation of mRNA processing or stability (Koushika et al., 1996; Beck et al., 1998; Fan & Steitz, 1998; Levy et al., 1998; Peng et al., 1998; Ford et al., 1999; Gueydan et al., 1999; Keene, 1999; Puig et al., 1999; Lambermon et al., 2000). Like plant RBP45 and RBP47, the metazoan and yeast proteins also contain auxiliary domains at their termini and/or in the region separating RBD2 and RBD3.

In vitro RNA-binding experiments performed with RBP45 and RBP47 indicated that both proteins have specificity for oligouridylates. Analysis of deletion mutants showed that a combination of any two RBDs was sufficient for strong and oligouridylate-specific interaction with RNA; binding of mutants containing RBD3, in combination with either RBD1 or RBD2, was also weakly competed by poly(G). Indirect immunofluorescence and cell fractionation experiments have shown that RBP45 and RBP47 are localized in the nucleus, where they are associated with the poly(A)$^+$ RNA, as demonstrated by the in vivo UV crosslinking. The domain organization, cellular localization, and RNA-binding properties (specificity for oligouridylates, association with nuclear poly(A)$^+$ RNA), make RBP45 and RBP47 very similar to UBP1, another *N. plumbaginifolia* protein characterized recently (Lambermon et al., 2000). Protoplast transfection experiments have indicated that UBP1 functions in a nuclear pre-mRNA maturation by stimulating splicing of suboptimal introns and increasing accumu-

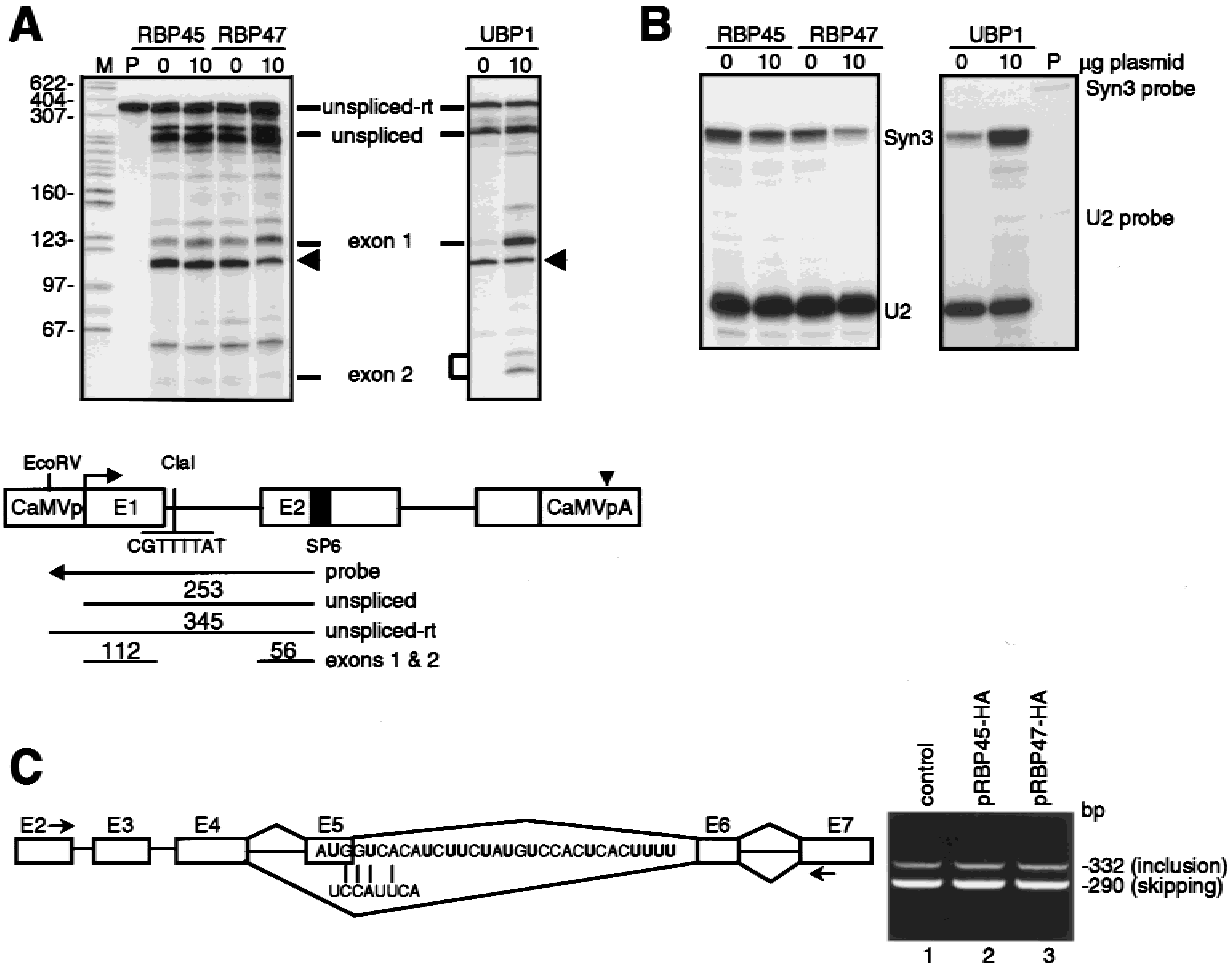


FIGURE 6. Effect of overexpression of RBP45 and RBP47 on splicing and RNA accumulation of reporter RNAs expressed in *N. plumbaginifolia* protoplasts. **A**: Analysis of splicing of SynGC/ClaU intron. Protoplasts were cotransfected with 5 μg of reporter plasmid (pSynGC/ClaU) with either 10 μg of the empty vector pDEDH/Nco (0) or with 10 μg (10) of the plasmid expressing RBP45-HA, RBP47-HA, or UBP1-HA. RNase A/T1 mappings were performed with the reporter-specific probe. Fragments diagnostic of spliced (exon1 and exon2) and unspliced RNAs are indicated. For the identity of the unspliced-readthrough (unspliced-rt) band (originating from the readthrough transcription going around the plasmid), see Lambermon et al. (2000). The band marked with an arrow represents the probe fragment protected by the readthrough transcript originating from plasmids pDEDH/Nco, pRBP45-HA, pRBP47-HA, and pUBP1-HA (Lambermon et al., 2000). Lane P: aliquot of undigested probe. Size markers (in nucleotides) are indicated. Scheme represents the SynGC/ClaU plasmid (Lambermon et al., 2000) with indicated sizes of RNA fragments protected by the probe. Transcription initiation and the poly(A) site are indicated by arrows. SP6 promoter used for antisense probe preparation is indicated by the black box. Insertion of a U-rich element in the intron at the *Cla*I site and the *Eco*RV site used for plasmid linearization for antisense transcription are also indicated. **B**: Effect of RBP45 and RBP47 on accumulation of intronless Syn3 RNA. Protoplasts were cotransfected with 5 μg of pSyn3 and either 10 μg of empty vector pDEDH/Nco (0) or 10 μg of the plasmid expressing RBP45-HA, RBP47-HA, or UBP1-HA (10). Mappings were performed with a mixture of Syn3 and U2 snRNA probes (U2 snRNA probe detects a 50-nt 5′-terminal fragment of endogenous U2 snRNA; Lambermon et al., 2000). Lane P: aliquot of undigested probes. **C**: Effect of RBP45 and RBP47 on splicing of the *ap3-1* reporter. Schematic representation of the *ap3-1* transcript with the indicated 5′ss region of intron 5 and its potential base pairing with U1 snRNA. The A → U mutation at the −2 position of the 5′ss is indicated in bold. Arrows indicate positions of oligonucleotide primers used for RT-PCR. The normal and the exon 5 skipping splicing patterns are indicated. The panel on the right shows the result of RT-PCR performed with RNA isolated from protoplasts transfected with *ap3-1* reporter plasmid alone (lane 1) or from protoplasts cotransfected with the plasmid expressing either RBP45-HA (lane 2) or RBP47-HA (lane 3). The product of 332 bp contains exon 5, whereas the product of 290 bp results from exon 5 skipping.

lation of reporter mRNAs that contain inefficiently processed introns or are intronless (Lambermon et al., 2000). Similar experiments, performed in this work with RBP45 and RBP47, have demonstrated however that these proteins do not affect splicing and accumulation of reporter RNAs in plant protoplasts.

Despite a similar domain organization and N-terminal enrichment in glutamines, UBP1 and RBP45/RBP47 are not highly related at the primary structure level: their sequences are only 31/35% identical. Although RBDs of UBP1 are most similar to those of the metazoan TIA proteins and the yeast Pub1p (Lambermon

et al., 2000), the RBD2 and RBD3 domains of RBP45 and RBP47 are most closely related in sequence to the respective RBDs of the yeast U1 snRNP protein Nam8p. The yeast U1 snRNA is approximately four times longer than the metazoan U1 snRNA, and is associated with seven yeast-specific proteins, among them Nam8p, which have no counterparts among integral components of the U1 snRNP in higher eukaryotes (Gottschalk et al., 1998; Fortes et al., 1999). Puig et al. (1999) have recently shown that Nam8p plays a role in splicing of introns with a suboptimal 5′ss, by interacting with intronic sequences positioned downstream of this site. Interestingly, of the limited set of elements tested, the U-rich sequence placed in the 5′ss-proximal region of the intron was the strongest effector of Nam8p activity. The nucleotide-binding specificity of Nam8p has not been directly determined, but this result and similarity of Nam8p and plant RBP45 and RBP47 RBD domains suggests that Nam8p has a specificity for oligouridylates.

Lengths, sequences, and the predicted secondary structure of U1 snRNAs in plants are very similar to those of metazoan U1 snRNAs (reviewed by Solymosy & Pollak, 1993), and it is therefore unlikely that RBP45 and/or RBP47 represent equivalents of Nam8p, being structural components of the U1 snRNP in plants. Consistent with this, immunoprecipitation experiments, performed with the *N. plumbaginifolia* nuclear extracts, have to date failed to demonstrate interaction of RBP45 and RBP47 with U1 snRNP (Z.J. Lorković & W. Filipowicz, unpubl. results). Likewise, expression of RBP45 or RBP47 in the yeast Δ*nam8* strain was not able to complement for Nam8p-specific defects in splicing (O. Puig, B. Séraphin, Z.J. Lorković, and W. Filipowicz, unpubl. results). We have also tested whether overexpression of either RBP45 or RBP47 could stimulate splicing of suboptimal introns having certain features in common with introns targeted by Nam8p in yeast (Puig et al., 1999). The synthetic intron SynGC/ClaU has U-rich element positioned downstream of the 5′ss, whereas a natural *Arabidopsis apetala* gene intron five contains, in addition to the 5′ss-proximal intronic U-rich sequence, a mutation in the 5′ss, which destabilizes its base pairing with U1 snRNA (Fig. 6A,C). RBP45 and RBP47 did not stimulate processing of either of these introns (Fig. 6), nor did they stimulate processing of the SynGC/ClaU2 intron (Gniadkowski et al., 1996), which has two copies of the U-rich element present in SynGC/ClaU (data not shown). In addition, we have found that coexpression of RBP47-MS2 coat protein fusion with the SynGC plasmid containing an MS2 binding site positioned downstream to the 5′ss did not result in stimulation of splicing of the reporter intron (data not shown). In spite of these negative findings, there is an obvious parallel between the intron-sequence-mediated effect of Nam8p in yeast and effects of U-rich elements, exerted from upstream or other intron location, on splicing in plants (Goodall & Filipowicz, 1989; McCullough et al., 1993; Gniadkowski et al., 1996; Egoavil et al., 1997). Given this similarity, it remains an attractive possibility that plant Nam8p-like proteins contribute to the recognition of U-rich intronic elements.

Seguin et al. (1997) have recently identified the RBP47-like protein in *N. tabacum* as a factor interacting with double-stranded DNA. The recombinant tobacco protein was shown to bind to the AC-rich promoter element in the phenylalanine ammonia-lyase gene (Seguin et al., 1997). The significance of this interaction is not clear. We have found (Z.J. Lorković & W. Filipowicz, unpubl. results) that purified recombinant *N. plumbaginifolia* RBP47 interacts with single-stranded oligouridylates more efficiently than with the DNA promoter element studied by Seguin et al. (1997). The N-terminal region of RBP45 and RBP47 is rich in glutamines, a feature also characteristic of activation domains in mammalian transcription factors such as Sp1, Oct1, and Oct2 (Escher et al., 2000, and references therein). However, we have found that fusion of the glutamine-rich domain of either RBP45 or RBP47 to the DNA-binding domain of the transcription factor GAL4 does not result in a protein able to activate transcription of appropriate reporter genes in transfected HeLa cells (O. Georgiev, W. Schaffner, G. Simpson, and W. Filipowicz, unpubl. results). The results of in vivo crosslinking experiments (Fig. 4) also argue in favor of RBP47 interacting with pre-mRNA rather than DNA in nuclei of *N. plumbaginifolia*. However, because some hnRNP proteins are known to contribute to transcription initiation (reviewed by Krecic & Swanson, 1999), it is conceivable also that RBP47 functions in both transcription initiation and pre-mRNA maturation.

We have also investigated the possibility that RBP45 and RBP47 may function in mRNA export and shuttle between the nucleus and the cytoplasm. In yeast and mammalian cells, shuttling of many nuclear RNA-binding proteins between the two compartments requires ongoing transcription, and inhibition of RNA synthesis with actinomycin D leads to the accumulation of the proteins in the cytoplasm (reviewed by Michael et al., 1995; Nakielny et al., 1997; Izaurralde & Adam, 1998). We have found that treatment of plant cells with actinomycin D does not change the cellular distribution of RBP45 and RBP47 proteins. Because some nuclear RNA-binding proteins continue to shuttle even when transcription is inhibited (reviewed by Michael et al., 1995; Nakielny et al., 1997; Izaurralde & Adam, 1998; Calado et al., 2000), heterokaryon fusion experiments are required to establish whether RBP45 and RBP47 move between the nucleus and the cytoplasm in plant cells.

In addition to UBP1 (Lambermon et al., 2000), RBP45, RBP47, and RZ-1 (this work; Fig. 4) are at present the only characterized plant proteins for which an interaction with nuclear poly(A)$^+$ RNA in vivo has been directly demonstrated. Hanano et al. (1996) have shown that RZ-1, a tobacco nuclear protein containing one RBD domain, sediments on glycerol gradients as part

of a 60S complex of unknown identity. By performing UV crosslinking experiments in vivo, we have demonstrated that RZ-1 is associated with poly(A)$^+$ RNA in the nucleus (Fig. 4). These proteins can therefore be classified as hnRNP-like proteins. Previous experiments suggested that nuclear extracts from plant cells contain few proteins interacting with pre-mRNA transcripts in vitro (Gniadkowski et al., 1996). However, results discussed above and database searches indicate that a complex set of hnRNP-like proteins, including proteins related to metazoan hnRNP A/B, I (PTB), and H/F, is expressed in plant cells (reviewed by Lorković et al., 2000). Interestingly, most of the investigated plant hnRNP-like proteins, such as UBP1 (Lambermon et al., 2000), UBP1-interacting RNA-binding protein UBA1 (M.H. Lambermon and W. Filipowicz, unpubl. results), RGP-3 (Moriguchi et al., 1997), PTB (Lorković et al., 2000), and RBP45 and RBP47 (this work) show affinity for oligouridylates. These findings, and observations that at least three UBP1 (Lambermon et al., 2000), three UBA1 (M.H. Lambermon and W. Filipowicz, unpubl. results), and seven RBP45 and RBP47 (this work) isoforms are expressed in *A. thaliana*, suggest that recognition of U-rich sequences present in plant introns and 3′-UTRs is a subject of a very elaborate regulation, likely to play an important role during gene expression in plants.

MATERIALS AND METHODS

Isolation of cDNAs encoding RBP45 and RBP47

Screening of the λ-ZAPII cDNA library of *N. plumbaginifolia* was as described by Mieszczak et al. (1992). By using RBD 2 (RBP45), and RBD 7 and 9 (RBP47) as probes (Mieszczak et al., 1992), we isolated 14 RBP45, and 2 RBP47 clones. The longest cDNA inserts chosen from the overlapping clones, referred to as pRBP45-SK and pRBP47-SK, were sequenced (Mieszczak et al., 1992). The sequences were analyzed using the Genetics Computer Group (GCG, Madison, Wisconsin) software package. Sequences of the *N. plumbaginifolia* RBP45 and RBP47 cDNAs are deposited at the European Molecular Biology Laboratory database (accession numbers AJ292767 and AJ292768, respectively). *A. thaliana* ESTs with high sequence similarity to RBP45 and RBP47 were obtained from the Arabidopsis Biological Resource Center, The Ohio State University. Complete sequences of *A. thaliana* RBP45 and RBP47 proteins were deduced from sequenced EST clones and the genomic DNA sequences that became available in the *A. thaliana* database. Unless indicated otherwise, all DNA manipulations were carried out according to Sambrook et al. (1989).

Plasmid constructions

Influenza hemaglutinin tag fusions

For expression of the RBP45-HA and RBP47-HA fusion proteins in protoplasts, pGGS5, a plant expression vector designed for expression of C-terminal HA tag (MYPYDVPDYA) fusions (Genschik et al., 1997) was used. To obtain RBP47-HA, the coding region of RBP47 in pRBP47-SK was PCR amplified with oligonucleotides TGCGG**GTCGAC**AATAAAC CATGAACGGAGGAGATATGAATC and TATTGC**AGATCT**G CTAACAGGCTGCTGGTGATTC, which introduce a *Sal*I site and a plant translation initiation site consensus sequence (Gallie, 1993), and a *Bgl*II site in place of the stop codon, respectively. The PCR product was cut with *Sal*I and *Bgl*II restriction enzymes and ligated into *Sal*I and *Bam*HI sites of pGGS5. To obtain pRBP45-HA, the coding region of RBP45 was PCR amplified using pRBP45-SK as a template, and KS universal oligonucleotide (Stratagene) and oligonucleotide CAACAGACGTCATATGGGTACTGCTGTGGTTGCTG complementary to the 3′ portion of cDNA as primers. The amplification product was digested with *Eco*RV and *Nde*I and ligated into *Sma*I and *Nde*I sites of pHAT0, another vector for expression of HA-tagged proteins. To construct pHAT0, *Nde*I and *Aat*II restriction sites in the pTTO vector (Mieszczak et al., 1992) were deleted, and the HA tag introduced in place of the tenascin tag, using *Bam*HI and *Xba*I restriction enzymes.

GST fusion plasmids

The coding regions of RBP45 and RBP47 were PCR amplified from the original cDNA clones in pBSC-SK using gene-specific oligonucleotides that introduce *Eco*RI sites replacing start and stop codons, respectively. All deletion mutants (except mutant that expresses RBD1 and RBD3) were amplified with oligonucleotides that introduced *Eco*RI sites before and after amino acids indicated in Figure 5B. PCR amplification products were cut with *Eco*RI and ligated into the corresponding site of pGEX-2T (Pharmacia). The mutant expressing RBD1 and RBD3 was constructed by cloning of RBD1 as a *Bam*HI-*Eco*RI fragment into pGEX-2T and subsequent ligation of RBD3 into the *Eco*RI site. All plasmids were sequenced before transformation and overexpression in *E. coli*.

Apetala3-1 reporter plasmid

The *apetala3-1* locus was PCR amplified from plasmid pD1103 (a kind gift from Thomas Jack, Dartmouth College, Hanover, New Hampshire) using oligonucleotides ATCAT**GTCGAC**AA AAAGATTAAACAAAGAG and CAGTA**GGATCC**TTCAAGAA GATGGAAGGTA. The PCR product was cut with *Sal*I and *Bam*HI and ligated into corresponding sites of pDEDH/Nco (Lambermon et al., 2000).

Overexpression of proteins in *E. coli*

RBP45 and RBP47 and their deletion mutants were expressed as N-terminal GST fusions in *E. coli* strain DH5α. Purification was done as previously described for GST-U2AF^{65} (Domon et al., 1998). After elution from glutathione-Sepharose 4B, proteins were concentrated using Millipore Ultrafree-4 centrifugal filters (Millipore); in parallel the buffer was exchanged into 20 mM HEPES-KOH, pH 8.0, containing 100 mM KCl, 0.2 mM EDTA, and 1 mM DTT.

Preparation of antibodies

BALB/c mice were immunized as described by Spitz (1986), using 50 μg of the recombinant RBP47 protein. Fusions were performed according to Lane et al. (1986). Hybridomas were grown in DME medium supplemented with 10% FCS (Harlow & Lane, 1988). Antibody titers were estimated using the IsoStrip Kit (Boehringer). To prepare a polyclonal antibody against RBP45, its C-terminal synthetic peptide, YYGNYPGY ANYQQPQQ, was coupled to KLH with glutardialdehyde and used for immunization of rabbits. For purification of the antibody, the peptide used for immunization, containing an additional cysteine at the C terminus, was coupled to SulfoLink® Coupling Gel (Pierce) according to the manufacturer's instructions. The bound antibody was eluted with 100 mM glycine, pH 2.2.

Western blotting

Proteins were separated by SDS-PAGE on 10–12% gels and electroblotted onto a PVDF Immobilon-P (Millipore) membrane. Antibodies were diluted as follows: α-RBP47 mAb RB4, 1:100; α-RBP45 polyclonal antibody, 1:2,000; α-U2B″ mAb 4G3 (Cappel), 1:100; mouse α-HA mAb 12CA5 (Boehringer), 1:100; rat α-HA mAb 3F10 (Boehringer, 50 μg/mL), 1:400. Secondary antibodies, all horseradish peroxidase conjugated, were diluted as follows: goat α-mouse antibody (Jackson ImmunoResearch Laboratories) 1:5,000; donkey α-rabbit antibody (Amersham), 1:10,000; and goat α-rat (Jackson ImmunoResearch Laboratories), 1:10,000. The blots were developed using the enhanced chemiluminescence (ECL) kit, according to the manufacturer's instructions (Amersham).

Growth of plant cells, protoplast preparation, cellular fractionation, indirect immunofluorescence and in vivo UV-crosslinking

Conditions for growth of plant cells and protoplast preparation were as described by Goodall et al. (1990). Cellular fractionation, indirect immunofluorescence and in vivo UV crosslinking were performed as described by Lambermon et al. (2000).

In vitro transcription, UV RNA–protein crosslinking and nitrocellulose filter binding assay

In vitro transcription by T7 or SP6 RNA polymerases, using [α-^{32}P]-UTP (800 Ci/mmol), and purification of labeled RNAs were performed as described earlier (Goodall et al., 1990). The in vitro crosslinking/ribohomopolymer-competition assays with the *rca* intron were as described by Domon et al. (1998).

For K_d determination, increasing amounts of recombinant proteins were incubated with the in vitro-transcribed, [α^{32}P]-UTP-labeled RNA under the same conditions as used for UV-crosslinking. The reactions were made to 100 μL with the binding buffer and immediately filtered through nitrocellulose filters (HAWP02500, 0.45 μm; Millipore) prewetted with binding buffer. After washing with 2.5 mL of binding buffer, filters were air dried and radioactivity was determined by Cerenkov counting.

Protoplast transfection and RNA analysis

Protoplast transfections and RNaseA/T1 protection assays were performed as described previously (Goodall et al., 1990; Lambermon et al., 2000). Because the *ap3-1* is a temperature-sensitive mutation (Sablowski & Meyerowitz, 1998), protoplasts transfected with this mutant were kept at 26 °C, the nonpermissive temperature. Analysis of *ap3-1* splicing was performed by RT-PCR. Three hundred nanograms of the total RNA isolated from transfected protoplasts was used for reverse transcription that was performed according to the manufacturer's instructions (Clontech). Subsequent PCR reactions were done according to Yi & Jack (1998), using TTGGGCCACTCAATATGAG and GAACTGAGTCGTAATC TCC oligonucleotides as primers. PCR products were analyzed on 2.5% agarose gels.

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