Translational control in plant stress: the formation of messenger ribonucleoprotein particles (mRNPs) in response to desiccation of *Tortula ruralis* gametophytes

Andrew J. Wood¹ and Melvin J. Oliver*

*Plant Stress and Water Conservation Unit, USDA-ARS, Route 3, Box 215, Lubbock, TX 79401, USA*

Summary

Changes in gene expression observed in vivo in response to desiccation and rehydration of the desiccation-tolerant bryophyte *Tortula ruralis* are regulated by alterations in the pattern of selection of messenger RNAs, from a qualitatively constant mRNA pool, by the translational machinery. When drying rates are slow, messenger ribonucleoprotein particles (mRNPs) are formed in the drying gametophytes. A representative rehydrin mRNA, Tr288, was sequestered into these particles which were analysed using sucrose and CsCl gradients. Quantitative RT–PCR analysis of the fractions from a low salt extraction demonstrated that Tr288 mRNA migrated farther in the sucrose gradient, relative to those extractable in high salt, indicating that the transcript is associated with particles that are of higher density. RT–PCR analysis also demonstrated that the majority of Tr288 mRNA, from slowly desiccated gametophytes, is associated with particles that have buoyant densities between 1.44 and 1.64 g cm⁻³ which correspond to the buoyant density range reported for mRNP particles. mRNPs that are unique to drying *T. ruralis* gametophytes form at least four size classes after in vivo UV cross-linking based upon FPLC analysis. This is the first report of mRNP formation in response to a vegetative water deficit in plants.

Introduction

Desiccation tolerance is defined as the ability to revive from the air-dried state (Bewley, 1979), which is the severest water stress since under these conditions the majority of protoplasmic water is lost from the cell. Most higher plants are capable of producing structures that are desiccation-tolerant (e.g. seeds and pollen), but only a few can survive the desiccation of their vegetative tissues. Desiccation-tolerant plants can be classified into those whose internal water content rapidly equilibrates to the water potential of the environment and modified desiccation-tolerant plants that employ morphological and physiological mechanisms to retard and control the rate of water loss (Oliver and Bewley, 1997). The ability of desiccation-tolerant bryophytes to survive relatively rapid desiccation requires the establishment of a rapid response mechanism. Studies into the response to desiccation and rehydration of the desiccation-tolerant moss *Tortula ruralis* have lead to the conclusion that this bryophyte has a constitutive protection system and a very active rehydration-induced recovery (repair?) mechanism (Bewley and Oliver, 1992; Oliver and Wood, 1997; Oliver, 1996). This is in contrast to the overall strategy proposed for the modified desiccation-tolerant angiosperm *Craterostigma plantagineum* which utilises a drying induced elevation of abscisic acid to trigger the accumulation of gene products that mediate the establishment of a cellular protection system prior to desiccation (Ingram and Bartels, 1996; Oliver and Bewley, 1997).

The rapidity of the response of *T. ruralis* to desiccation and rehydration has been demonstrated in studies into the regulation of gene expression during these events. Early work firmly established the capability of *T. ruralis* to rapidly recover synthetic metabolism upon rehydration following a drying event (Bewley and Oliver, 1992; Bewley, 1979). In the period immediately following rehydration, desiccated *T. ruralis* produces a set of polypeptides whose synthesis is unique to the rehydrated state. Although the pattern of protein synthesis within the first two hours of rehydration is distinctly different from that of hydrated controls, novel transcripts were not synthesised during desiccation or upon rehydration (Oliver and Bewley, 1984b; Oliver and Bewley, 1984c; Oliver, 1991; Scott and Oliver, 1994). These observations lead to the hypothesis that in a bryophyte that has to respond rapidly to desiccation and rehydration, an alteration in translational control is used to modify gene expression. Such a mechanism would allow for the rapid synthesis of proteins that mediate the survival of the desiccated vegetative cells once water is returned to the plant.

The complexity of the translational controls that operate during a desiccation/rehydration event was demonstrated in a detailed study of the changes in protein synthesis initiated by rehydration of dried and partially dehydrated *T. ruralis* gametophytes (Oliver, 1991). During the first two
hours of hydration following a rehydration event, the synthesis of 25 proteins is terminated (or substantially decreased) and the synthesis of 74 proteins is initiated (or substantially increased). The altered synthesis of these two groups of proteins, designated hydrins and rehydrins, respectively, is not co-ordinately regulated (Oliver, 1991).

Rehydrin synthesis is initiated or stimulated upon rehydration of gametophytes that had been previously dried to between 50 and 20% of their fresh weight, while the synthesis of hydrins is inhibited in gametophytes that were previously dried to 50% of their fresh weight. These findings indicate a threshold of prior water loss exists at which the protein-mediated portion of the recovery mechanisms is fully activated. This in turn suggests the existence of a mechanism by which the amount of water loss is translated into a protein synthetic response. Hydrins and rehydrins also differ in the time required to return to normal levels of synthesis during extended hydration. The synthesis of all hydrins returns to control levels between 2 and 4 h following rehydration. The reduction in synthesis of rehydrins to control levels depends upon each individual rehydrin. Some rehydrins are only synthesised transiently, while others are still being synthesised at elevated levels 10–12 h after hydration. A full return to control levels of synthesis for all proteins is evident 24 h post-hydration. The terms hydrin and rehydrin are empirical definitions and thus do not refer to any common sequence motif or structural property nor imply a common enzymatic function.

Scott and Oliver (1994) isolated 18 separate rehydrin cDNAs and 22 hydrin cDNAs. As predicted from the in vitro protein synthesis studies (Oliver and Bewley, 1984c; Oliver, 1991), rehydrin transcripts are not unique to rehydration. All 18 rehydrin cDNAs represent mRNAs present in hydrated moss cells but are present in greater amounts in the polysomes of rehydrated gametophytes compared with those from undesiccated moss. Utilising both hydrin and rehydrin cDNAs as probes, it was determined that during slow drying rehydrin cDNAs accumulate, even though they are not translated at this time, and that this accumulated RNA can be fractionated into the 100K g pellet of cellular extracts (Oliver and Wood, 1997; Oliver, 1996). The 100K g pellet (polysomal fraction) contains most of the cytosolic translational machinery (such as RNA-protein complexes, ribosomes, polysomes, etc.) as well as other cellular constituents which have sufficient mass to pellet through a 1.5-M sucrose pad at 100K g over 90 min. Hydrin cDNAs do not exhibit this behavior (Oliver and Wood, 1997; M.J. Oliver, unpublished data). This accumulation of transcripts in the 100K g pellet during drying occurs when gametophyte polysomes have been fully depleted and protein synthetic activity is completely inhibited (Bewley and Oliver, 1992; Oliver and Bewley, 1997; Oliver, 1996). This suggests the formation of protein/mRNA complexes, termed messenger ribonucleoprotein particles (mRNPs), that form large enough structures to sediment in the polysomal fraction during centrifugation. Such particles, first described by Spirin et al. (1964) studying fish and sea urchin embryo cytoplasmic extracts, are common in eukaryotic systems (Bag, 1988; Davidson, 1986). In plants their formation has been demonstrated in desiccated tissues, viz., seeds (Pramanik et al., 1992; Silverstein, 1973). In this report we describe the isolation and characterisation of the storage form of one rehydrin mRNA that forms during desiccation and our approach to identify the components that direct this storage.

Results

Identification of mRNPs in desiccating Tortula ruralis

Tr288 (GenBank accession number U21679) is a rehydrin (Scott and Oliver, 1994) that codes for an unidentified protein that has some similarity in primary structure to higher plant Lea proteins (Close et al., 1993). This rehydrin has a transcript that is abundant during slow drying and, as is typical of all rehydrins tested, accumulates to a high degree in the polysomal fraction of cellular extracts from slow-dried gametophytes (Oliver and Wood, 1997). Such properties make Tr288 an ideal choice for use as a marker for rehydrin transcript accumulation in storage particles during drying.

In order to determine if mRNP formation is an important aspect of the response to desiccation, we analysed the 100K g pellet from gametophytic extracts on sucrose density gradients (Figure 1). The sucrose gradient was fractionated from the top and the polysomal profile obtained from control, fully hydrated moss gametophytes, isolated under conditions of low salt (60 mM KCl), is shown in Figure 1(d). Polysomes were present in fractions 9–20 (i.e. mid to bottom of the gradient) and represent the active translational machinery of the normal, hydrated state. The hydration polysomal profile was not altered by the isolation of the polysomal pellet under high salt conditions (200 mM NaCl; Oliver and Bewley, 1984a) as was also true for the slow-dried polysomal profiles (compare Figure 1e and f). The sucrose gradients were further analysed for the presence of an abundant rehydrin Tr288 as a marker of rehydrin transcripts (Scott and Oliver, 1994) using RT–PCR (Figure 1a,b,c). Although abundant following rehydration and during slow desiccation, the Tr288 transcript accumulated to only very low levels under normal hydrated conditions as can be seen in Figure 1(a–c) and in previous work (Scott and Oliver, 1994). Nevertheless, the Tr288 transcript is detectable in the control gradient and is associated with the polysomal fractions indicating its translatability (Figure 1a). The full-length nature of Tr288.
mRNA was confirmed by Northern blot analysis of the 100K g pellet prior to sucrose/CsCl gradient fractionation and by RNA dot-blot analysis of the sucrose gradient fractions using a Tr288 cDNA probe. Each gradient fraction was spiked with 20 ng of DNruI-Tr288 mRNA and subjected to RT-PCR using gene-specific primers. Tr288 levels are expressed in ‘relative units’ normalised to the DNruI-Tr288 internal standard. (d–f) Polysome profiles of the 100K g pellet were generated and analysed as described in Experimental procedures.

When the polysomal pellets from slow-dried moss gametophytes, extracted in either high or low salt, were fractionated (Figure 1e,f) the resulting profiles were devoid of peaks in the region of the gradient that delineates polysomal complexes (fractions 9–20). As can be seen in Figure 1e,f, the loss of polysomes was reflected in an increase in the relative amount of ribosomal subunits and monosomes. Treatment of the 100K × g pellet with RNase prior to polysome analysis by sucrose gradients did not alter the polysome profile (Bewley, 1972). The loss of polysomes in response to slow desiccation is a well-documented phenomenon in T. ruralis and is a manifestation of ribosomal run-off from mRNAs concomitant with a deficiency in the re-initiation pathway (see Bewley, 1979; Bewley and Oliver, 1992; Bewley and Krochko, 1982; for reviews).

RT-PCR analysis of the polysomal fractions from a low salt extraction demonstrates that the Tr288 mRNA migrates farther in the sucrose gradient than Tr288 mRNA from a high salt extraction (Figure 1a–c). This indicates that the transcript is associated with particles that have a higher density than those extractable in high salt. The accumulation of Tr288 transcript in polysomal pellets of slow-dried moss is not unique, all of the rehydrin transcripts tested so far behave in this fashion but to varying degrees (Oliver and Wood, 1997; M.J. Oliver, unpublished data). Only rehydrin transcripts accumulate in polysomal fractions during slow desiccation.

Analysis of stored rehydrin mRNA using CsCl density gradients

Although the sucrose density analysis of polysomal pellets indicates the presence of large complexes that contain rehydrin transcripts it does not provide any information on their possible composition. In order to address this question the polysomal pellets were analysed by centrifugation within CsCl gradients which allows for the determination of the buoyant densities of the complexes identified in the sucrose density analysis. The buoyant density of a complex or particle is determined by its composition and, by controlling the range of densities that the gradient can encompass, the analysis can be directed towards the analysis of RNA and protein complexes specifically. The results of this type of analysis are presented below.

Traditionally, ribonucleoprotein complexes have been analysed and classified by virtue of their buoyant density in CsCl density gradients after the proteins and nucleic acids have been cross-linked (Bag, 1988). In this study, gametophytes were irradiated with short wave UV as a cross-linking procedure. UV irradiation covalently links
only those structures or molecules that are in direct contact (i.e. it is considered a zero-length cross-link) and prevents the dissociation of mRNP complexes during extraction (Bag, 1988; Bag, 1991). Cross-linking, coupled with the addition of 5 M urea to the CsCl gradients, ensures the isolation of mRNP complexes without the non-specific binding of proteins to mRNA (Dreyfuss, 1986; Greenburg, 1980).

The CsCl/5 M urea gradient profiles for the 100K g pellets from low salt extracted, UV-treated (i.e. in vivo cross-linking) control and slow-dried gametophytes are presented in Figure 2(a). Relatively shallow gradients, 1.2–1.7 g cm⁻³, were used to accentuate the range of buoyant densities revealed for cross-linked mRNPs (1.4–1.5 g cm⁻³, Bag, 1988). In the gradients for both control and slow-dried samples, the bulk of the material resided in the fraction delineated by buoyant densities of 1.3–1.4 g cm⁻³. It is assumed components in this range are either pure protein or RNA/protein complexes that have relatively high protein to RNA mass ratios since the buoyant density of pure protein ranges from 1.2 to 1.36 g cm⁻³, depending upon the degree of hydration (Hamilton, 1967). The reported buoyant density of free RNA is 1.7–1.9 g cm⁻³ (Bag, 1988; Hamilton, 1967; Spirin, 1969). Ribosomal subunits are reported to have buoyant densities of 1.55–1.59 g cm⁻³ when isolated after formaldehyde treatment (Spirin, 1969). Both gradients contained a significant amount of material in the 1.4–1.5 g cm⁻³, fractions that were less dense than the ribosomal subunits and correlate with protein/RNA complexes.

To determine if RNA was indeed being stored as a protein/RNA complex during a drying event, moss gametophytes were preincubated with 3H-uridine and 35S-methionine/cysteine. The 100K g pellet was fractionated in a CsCl-Urea gradient (approximately 1.2–1.7 g cm⁻³ CsCl; 5 M urea) and the distribution of dual radiolabel was determined using a liquid scintillation analyser as described. Two independent experiments were performed and essentially the same results were obtained each time.

Figure 2. Density analysis of the 100K g pellet from control and slow-dried gametophytes subjected to in vivo UV-cross-linking. (a) The 100K g pellet was isolated using the low-salt extraction buffer and 10 OD260 units were fractionated in a CsCl-Urea gradient (approximately 1.2–1.7 g cm⁻³ CsCl; 5 M urea) and separation of the components was monitored by UV-absorbance at 260 nm. The direction of centrifugation was from left to right. Prior to polysome isolation, mRNPs were stabilised in vivo by exposure to UV-irradiation as described in Experimental procedures. (b) Moss gametophytes were preincubated with 3H-uridine and 35S-methionine/cysteine as described in Experimental procedures and mRNPs were stabilised in vivo by UV-irradiation. The 100K g pellet was fractionated in a CsCl-Urea gradient (approximately 1.2–1.7 g cm⁻³ CsCl; 5 M urea) and the distribution of dual radiolabel was determined using a liquid scintillation analyser as described. The dashed lines delimit the region of the gradient indicative of a drying induced binding of protein to RNA that results in a decrease in the buoyant density of the RNA. ○ 35S-methionine/cysteine; ● 3H-uridine. Two independent experiments were performed and essentially the same results were obtained each time.

Figure 3. Density analysis of the 100K g pellet from control and slow-dried gametophytes in the absence of in vivo UV-cross-linking. The 100K g pellet was isolated using the low-salt extraction buffer and 10 OD260 units were fractionated in a CsCl-Urea gradient (approximately 1.2–1.7 g cm⁻³ CsCl; 5 M urea). The gradient was collected from the top and separation of the components was monitored by UV-absorbance at 260 nm. The direction of centrifugation was from left to right. Two independent experiments were performed and essentially the same results were obtained each time.
not evident in the control gradient. This was indicative of a drying induced binding of protein to RNA that results in a decrease in the buoyant density of the RNA.

The results of the 35S-protein labelling are not so clear. As expected, the majority of the 35S label is contained in the upper fractions of the gradient that corresponded to pure proteins, i.e. 1.2±1.35 g cm$^{-3}$ (Hamilton, 1967), only a relatively small portion of the label was contained in the 1.4±1.50 g cm$^{-3}$ fraction. Drying of the gametophytes did not result in a significant shift in the buoyant density of the 35S-labelled protein that would indicate an increase in binding to RNA.

Quantitative RT–PCR of mRNP associated mRNA

To determine if rehydrin transcripts are associated with proteins in the complexes that have buoyant densities in the range of 1.4–1.5 g cm$^{-3}$, RNA was purified from these fractions and probed for the presence of the marker rehydrin, Tr288, using RNA gel blot analysis (data not shown). Hybridization to full-length, undegraded transcript was detected in fractions 10–20 which indicated that Tr288 mRNA was present in fractions of intermediate density. However, it is extremely difficult to quantify the level of target RNA in these fractions by Northern analysis since normal internal marker RNAs are neither present in these fractions (e.g. ribosomal RNAs) or are altered by the desiccation event (Oliver, 1991). Such quantification is essential if it is to be determined if rehydrins are preferentially sequestered in protein/RNA complexes during slow drying. To overcome these problems, quantitative RT–PCR (Murphy and Taiz, 1995) was employed to follow the partitioning of a representative rehydrin, Tr288. Standard curves were generated, using Tr288 and ΔNruI-Tr288 cDNA as template for PCR, to ensure that quantitative comparisons occur within a linear range of DNA amplification (data not shown).

The use of an RT–PCR based assay for rehydrin transcripts required that the polysomal pellets used in the CsCl/5 M urea gradients were derived from gametophytes that had not been subjected to UV cross-linking. UV cross-linking of proteins to RNA, though it allows primer binding (see below), may prevent an accurate quantification of RNA in individual samples and thus render sample to sample comparisons meaningless. The CsCl/5 M urea gradient profiles from control and slow-dried non-cross-linked polysomal pellets are presented in Figure 3. As expected there was a shift of material in both profiles towards the more dense components of the pellets indicating an increase in free RNA. In particular there was an obvious peak of material at approximately 1.6 g cm$^{-3}$. Such a shift is indicative of a loss of protein from the RNA-protein complexes. Nevertheless, there was still a significant amount of material in the 1.4±1.5 g cm$^{-3}$ density range, similar to that seen in the gradient analysis of the UV crosslinked samples (Figure 3a), indicating that protein/RNA complexes resistant to high ionic strength conditions remain in the gradient.

Each gradient from Figure 3 was divided into five fractions based upon buoyant density for use in the RT–PCR assay. The pooled fractions represent densities of: fraction 1, 1.2–1.34 g cm$^{-3}$; fraction 2, 1.35–1.43 g cm$^{-3}$; fraction 3, 1.44–1.52 g cm$^{-3}$; fraction 4, 1.53–1.64 g cm$^{-3}$; fraction 5, 1.65–1.8 g cm$^{-3}$. Each pooled fraction was spiked with a fixed quantity of an in vitro synthesised Tr288 mRNA that contained a 61-bp deletion in the region that is delineated by the primers used in the RT–PCR assay (ΔNruI-Tr288).


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mRNPs were isolated from the resulting 100K g methionine/cysteine as described in Experimental procedures. Polysomal moss gametophytic tissue was preincubated with 3H-uridine and 35S-gametophytic tissue in the absence and presence of UV-irradiation. Figure 5. Isolation of mRNP complexes from control and slow-dried 364 Andrew J. Wood and Melvin J. Oliver cm fractions of intermediate buoyant density, i.e. 1.35±1.52 g gametophytes the majority of the Tr288 mRNA was in density). In the polysomal pellet from slowly desiccated buoyant density) or relatively protein free (high buoyant protein/RNA complexes that are very rich in protein (low buoyant density (fractions 1) and relatively high buoyant density fractions indicating a large amount of relatively protein-free Tr288 mRNA from the polysomal pellet of slow-dried non-cross-inked gametophytes.

Isolation of polysomal mRNP complexes

The data presented above strongly indicate that rehydrin mRNAs are sequestered in mRNP particles during the slow desiccation of T. ruralis gametophytes. Although CsCl gradients are useful for profiling RNA/protein complexes, they are of limited use for isolation of specific particles such as mRNPs. A more useful approach is to directly isolate mRNPs by oligo-(dT) chromatography, a technique that has allowed for the complete characterisation of mRNPs in animal systems (Bag, 1988; Minich et al., 1975). Such characterisations frequently make use of the ‘zero-length crosslink’ property of UV irradiation to stabilise the particles during purification and limit the isolation of proteins that are only peripherally attached to the complexes. The isolation of mRNP complexes under similar conditions has been well documented (Bag, 1988; Minich and Ovchinnikov, 1992).

Polysomal mRNP particles were isolated using oligo-(dT) chromatography (Bag, 1988; Jain et al., 1979). As in the previous experiment, as a means of tracking the two components, the gametophytes were incubated with 3H-uridine and 35S-methionine/cysteine for 36 h prior to drying and extraction. However, since the mRNP fractions of the CsCl gradients were not heavily labelled with 35S-methionine/cysteine (Figure 2), a much larger amount of gametophytic tissue (4 g instead of 0.5 g) was required in order to obtain significant levels of incorporation for this analysis. Following irradiation with UV light, the gametophytes were extracted and the 100K g pellet was incubated with DynaBeads oligo-(dT25) and protein/poly A + RNA complexes were subsequently purified. In order to directly compare samples and better track the involvement of protein with mRNA, the 35S-labelled protein associated with poly A + was normalised to the amount of 3H-labelled RNA co-purified. The results of such an isolation is presented in Figure 5. UV-irradiation allows the purifica- tion of 35S-labelled protein/poly A + RNA complexes in both control and slow-dried samples. However, there is a 2.5 fold increase in 35S-labelled protein associated with poly A + RNA following slow desiccation, that has previously shown to be a treatment when protein synthesis has ceased and polysomes are no longer present (Bewley, 1979; Bewley and Krochko, 1982; Oliver, 1991). In the absence of cross-linking, the association of the protein with the RNA is not fully stable to the conditions used for oligo-(dT) chromatography as is evidenced by the 10- and 50-fold less 35S-labelled protein in the non-UV irradiated control and slow-dried samples, respectively (Figure 5).
To further characterise the mRNP fractions isolated by oligo-(dT) chromatography, the pooled labelled poly A + RNA fractions from five separate isolations (a total of 22 gm of gametophytic tissue) were subjected to Fast Protein Liquid Chromatography (FPLC) using a gel filtration sizing column, superose 6 HR10/30. In the absence of cross-linking by UV-irradiation, 100K g pellets from control gametophytes contained a single complex that is contained in a peak with an elution volume of 19 ml (peak 1) (Figure 6a and Table 1). This peak contained significant levels of 3H poly A + RNA (14% of the total label) but negligible amounts of 35S-labelled protein. This indicated that this peak contained either free polysomal poly A + RNA that has been released during oligo-(dT) chromatography or pelletable poly A + RNA that is complexed to a protein that is not turned over during the pre-labelling period. This peak was present in all samples (Figure 6a–d and Table 1) regardless of the UV-irradiation treatment. The elution volume of this peak would represent a molecular mass of 20.8 kDa if poly A + RNA or a protein/ mRNA complex were to behave as a globular protein within the superose matrix. Since this is unlikely, such measurements are misleading. Nevertheless, we have included the molecular mass values for each peak to present some familiar relative measure of magnitude for each of the particles revealed in this analysis.

The 100K g pellets from UV-irradiated control gametophytes contained, in addition to the peak at 19 ml (120 kDa, peak 1), a smaller 35S-labelled protein/poly A + RNA complex that eluted at 27.5 ml (20.8 kDa, peak 2) (Figure 6c and Table 1). This peak contained 30% of the total 35S-labelled protein contained in the 100K g pellet in addition to 4% of the total 3H poly A + RNA (Table 1). This peak was also present in the pellet from slowly dried UV-irradiated gametophytes (Figure 6d and Table 1) and contained approximately 20% of the total 35S-labelled protein and 12% of the 3H poly A + RNA. However, in the pellet from slowly dried UV-irradiated gametophytes, several novel 35S-labelled protein/poly A + RNA complexes can be discerned (Figure 6d and Table 1). Three of these desiccation induced complexes eluted as very large entities with elution volumes of 9 (1275 kDa), 13 (550 kDa), and 16 ml (270 kDa), respectively. The remaining novel complex was intermediate in size between the two complexes observed.

**Table 1.** Amount of 3H-uridine and 35S-methionine/cysteine incorporated into oligo (dT)-purified mRNP particles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H-uridine dpm (% of total)</th>
<th>35S-met/cys dpm (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (minus UV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1 (19 ml)</td>
<td>275 (14%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Slow-Dried (minus UV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1 (19 ml)</td>
<td>262 (6%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Control (plus UV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1 (19 ml)</td>
<td>250 (6%)</td>
<td>40 (0.8%)</td>
</tr>
<tr>
<td>Peak 2 (27.5 ml)</td>
<td>150 (4%)</td>
<td>950 (30%)</td>
</tr>
<tr>
<td>Slow-dried (plus UV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1 (19 ml)</td>
<td>722 (26%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Peak 2 (27.5 ml)</td>
<td>310 (12%)</td>
<td>2220 (20%)</td>
</tr>
<tr>
<td>Peak 3 (22 ml)</td>
<td>170 (7%)</td>
<td>380 (4%)</td>
</tr>
<tr>
<td>Peak 4 (16 ml)</td>
<td>270 (10%)</td>
<td>730 (7%)</td>
</tr>
<tr>
<td>Peak 5 (9 ml)</td>
<td>110 (4%)</td>
<td>1100 (10%)</td>
</tr>
<tr>
<td>Peak 6 (13 ml)</td>
<td>210 (7%)</td>
<td>650 (6%)</td>
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</tbody>
</table>

*The amount of labelled material was determined by liquid scintillation counting.

The average background radiation was 48 dpm ± 5 dpm; this value was determined by counting blanks containing only the scintillation cocktail.
in the control UV-cross-linked sample; and has an elution volume of 22 ml (65 kDa). These complexes contained the majority of the labelled protein and poly A + RNA extracted in the pellet and recovered by oligo-(dT) chromatography. In the UV-cross-linked control 30% of the 35S-labelled protein and 4% of the 3H poly A + RNA coeluted as a discernible peak (peak 2). In the UV-cross-linked slow dried sample 47% of the 35S-labelled protein and 66% of the 3H poly A + RNA coeluted as a discernible peaks (peaks 2-6) and of this 27% of the 35S-labelled protein and 40% of the 3H poly A + RNA are contained in the novel peaks (peaks 3–6). This clearly demonstrates that during slow drying mRNA (poly A + RNA) is ‘sequestered’ into distinct messenger ribonucleoprotein particles (mRNPs).

To ascertain if rehydrin transcripts were associated with these peaks, samples were taken and subjected to RT–PCR to detect the marker rehydrin Tr288 as described earlier. Since the samples had been cross-linked, no attempt to quantify the transcripts were made and PCR amplification was extended to 30 cycles. The results of this analysis demonstrate that Tr288 transcript is present in all of the identified peaks (data not shown).

Discussion

The extensive early work concerning the response of Tortula ruralis to a desiccation/rehydration event has led to the conclusion that, unlike many other plant stress responses, the alteration in gene expression elicited by the stress is primarily regulated at the translational level (Oliver, 1991; Oliver, 1996; Oliver and Bewley, 1984c; Oliver and Bewley, 1997; Scott and Oliver, 1994). The alteration in protein synthetic patterns during rehydration (proteins are not synthesised during drying in T. ruralis) occurs as a result of differential selection and/or recruitment of rehydrin mRNAs from a qualitatively constant mRNA pool. The results of this study demonstrate that in moss that dries at ecologically significant rates (i.e. slowly), the selection and/or recruitment of rehydrin mRNAs is in part aided by the storage of these transcripts as mRNPs during drying.

The formation and existence of mRNPs during slow desiccation of T. ruralis is supported by several lines of corroborating evidence that is consistent with the descriptions of the behaviour and structure of mRNPs from other systems, both plant and animal. Sucrose density gradient analysis of alfalfa embryos, using a high-salt buffer identical to the one used in this study, has demonstrated that 11S, 7S and 2S storage proteins are present in a unique mRNP fraction that has a density less than that of ribosomal subunits (Pramanik et al., 1992). This result is directly comparable to the high salt sucrose density analysis of desiccated T. ruralis gametophytes using RT–PCR to detect the presence of Tr288 (Figure 1). Analysis of the polysomal pellet from dried gametophytes using low salt buffers suggests that the particles may aggregate into heavier particles, and, since they are disrupted by high salt, the aggregation may occur as a result of interionic bonding. It is also possible that the increase in sedimentation rate is indicative of binding of the particles to other cellular components. Whether or not the particles aggregate or if such aggregations (or associations) occur in vivo remain to be determined.

The sucrose gradient analysis does present another alternative. It is remotely possible that the Tr288 transcript is associated with a small population of polysomes (not evident in the OD254 profile) that is resistant to drying but disrupted by high salt extraction. The high-and low-salt polysome isolation buffers, with the exception of NaCl concentration (400 mM versus 0 mM), are biochemically similar. They produce similar polysome profiles and will solubilise virtually all polysomes regardless of their cellular source (Abe et al., 1992). Northern blot analysis using ribosomal probes (data not presented) does indicate that ribosomal RNA can be detected in the lower fractions of the low salt sucrose gradient. However, there is no ribosomal RNA associated with the fractions that contained the Tr288 transcripts from the high-salt analysis. Thus, if the Tr288 transcript is associated with a unique subset of polysomes, it is associated as a particle. Additional evidence that Tr288 transcript is not polysome associated was obtained using puromycin, a protein synthesis inhibitor which causes premature chain termination resulting in release of messages from polysomal complexes. Pre-incubation of moss gametophytes with puromycin does not prevent the localisation of Tr288 mRNA in the 100K g pellet from slow-dried gametophytes based upon RNA dot-blot analysis of the fractions using a Tr288 DNA probe that encompasses the 5’ UTR and translational start site (data not shown). Its localisation in the more dense fractions of the low-salt sucrose gradients suggests that the Tr288 transcript is present as a discrete particle. The existence of a separate particle is more strongly supported by the buoyant density and RT–PCR analysis shown in Figures 3 and 4. In this case the polysomal pellet was not cross-linked by UV-irradiation and polysomal complexes would be disrupted by passage into the cesium chloride gradient (Figure 3). The RT–PCR analysis, however, demonstrates that the Tr288 transcript was still associated with particles that have buoyant densities between 1.44 and 1.64 g cm$^{-3}$. Although unprecedented, and we feel unlikely, the possibility of a small, salt sensitive population of polysomes cannot be ruled out by our studies. The later buoyant density and oligo-dT-FPLC analyses (Figures 5 and 6) further weaken this possibility but do not fully negate it.

Based upon its sedimentation in sucrose density gradients and its buoyant density in CsCl density gradients, rehydrin Tr288 mRNA was associated with protein (which
is bolstered by the 35S methionine/cysteine labelling studies discussed below). Spirin (1969) demonstrated that the buoyant density of fixed mRNA-protein complexes in CsCl gradients ranged from 1.42 to 1.45 g cm$^{-3}$ and that ribosomal subunits ranged from 1.52 to 1.58 g cm$^{-3}$. In UV-cross-linked gametophytes (Figure 4), radiolabelled RNA and protein are present (albeit at low levels) in fractions with a buoyant density of between 1.4 and 1.5 g cm$^{-3}$. In non-cross-linked slow-dried gametophytes (Figures 3 and 6), Tr288 transcripts are located in the 1.35–1.52 g$^{-3}$ range indicative of their association with protein. In the absence of cross-linking, proteins are more easily dissociated from RNP$\text{s}$, and their density increases. This would explain the presence of higher density particles in the 1.53–1.64 g cm$^{-3}$ range in these samples; nevertheless, most of the Tr288 mRNA remained in the lower density fractions (2 and 3). Since densities can be altered by the extraction conditions and the hydration state of the proteins (Henshaw, 1979), the density of the particles seen in the analysis of slow-dried gametophytes is well within the reported densities typical of bulk mRNP (Bag and Sarkar, 1975; Spirin, 1969) from both animal and plant tissues and specific mRNPs which have been studied in greater detail (Bag, 1988; Iskakov and Madin, 1994; Minich and Ovchinnikov, 1992).

The formation of mRNPs that contain rehydrin transcripts during slow desiccation is also substantiated in experiments that utilise in vivo labelling of RNA and protein. In the cesium chloride gradient analysis of cross-linked polysomal pellets (Figure 2), slow desiccation resulted in a shift in the distribution of the 3H-labelled RNA, when compared to the hydrated control, into the region of the gradient that delineates cytoplasmic mRNPs (1.4–1.5 g cm$^{-3}$). Somewhat surprisingly, there was not a detectable associated shift in the distribution of the 35S-met/cys-labelled protein. However, when a much larger sample was used for the separation of mRNPs from polysomal pellets by oligo-dT chromatography, an increase in the cross-linked 35S-met/cys-labelled protein content in the fraction from slow desiccated gametophytes compared to control cross-linked samples was evident (Figure 5). The inference from these experiments is that the protein or proteins associated with the mRNPs in T. ruralis during desiccation are present in the hydrated state and are relatively stable protein(s) and slow to turn over. This would be expected for an organism that leads an opportunistic life style and must always be prepared for a desiccation event. It is also possible that the proteins are deficient in methionine and cysteine residues and therefore do not accumulate the label. This possibility remains to be explored once we are able to isolate sufficient quantities of protein from these mRNPs for further analysis.

The ability of mRNPs to bind to oligo-dT has been well documented and is a major defining factor in their isolation from many and varied systems (Bag, 1984; Bag, 1988; Bag, 1991). For desiccating T. ruralis gametophytes, the mRNP particles that bind to oligo-dT separate into several novel size classes (after cross-linking) that are not present in hydrated controls (Figure 6 and Table 1). Each peak contained Tr288 rehydrin transcript and, with the exception of peak 1, radiolabelled protein. The function of the various size classes of particles is unknown and it is entirely possible that they are simply the manifestation of variability associated with in vivo UV-cross-linking. Such determinations remain to be investigated. This experiment also demonstrated that there are mRNPs in hydrated gametophytes (Figure 6c), a not unexpected result, and that these particles also remain in the dried state. The fact that desiccation results in new particles indicates the possibility that the desiccation mRNPs are formed by the association of a desiccation-specific RNA binding protein with the rehydrin transcripts (see Oliver and Wood, 1997 for the rehydrin transcripts known to accumulate in the polysomal fraction during desiccation). We are attempting to investigate this possibility but it has proven difficult to isolate sufficient quantities of these particles for a detailed reproducible protein analysis because of the large quantities of gametophytic tissue required. Polypeptides associated with polysomal and free mRNP complexes have been described in a number of systems (Bag, 1988; Bag and Sells, 1981; Dreyfuss et al., 1988; Iskakov and Madin, 1994; MacDonald and Williams, 1992). Using in vivo cross-linked preparations of chicken muscle cells, Bag (1984) has demonstrated that the major polypeptides associated with both polysomal and free mRNPs have Mr that range from 150 000 to 28 000. The majority of proteins identified in non-cross-linked mRNP complexes are also found within the cross-linked mRNP complexes (Bag, 1984; Bag, 1988; Bag, 1991). These data indicate that although mRNP complexes are relatively stable during isolation, cross-linking allows the isolation of a more complete mRNP protein complement. Our data would support this finding, however, many of the earlier studies used formaldehyde as a cross-linking agent and thus effected the stabilisation of both loosely and strongly attached proteins. The more recent use of UV-irradiation only cross-links those proteins in close contact with the RNA molecules (Bag, 1988; Bag, 1991) and thus we would expect fewer proteins to be present. This has added to our difficulty in obtaining sufficient protein for analysis.

The formation of mRNPs in response to water loss and their possible roles in mRNA storage and protection has important consequences for the study of vegetative desiccation-tolerance and perhaps stress responses of plants in general. The ability to store components during a stress event that are needed for recovery offers a new dimension to the concept of damage control and the possibility for a more rapid return to growth than does the
relatively slower activation and transcription of specific stress or stress-recovery response genes. It is distinctly possible, even in plants where gene activation is the common response to water loss, that certain transcripts required for the recovery process are stored in mRNPs during drying. At the least, the determination that the moss _T. ruralis_ makes use of stored mRNAs during the recovery phase should indicate that not all transcripts made in response to a stress event are required for immediate use but may be synthesised and stored for use when the stress is over and recovery is set in motion.

**Experimental procedures**

**Plant material**

Gametophytes of _Tortula ruralis_ [Hedw.] Gaertn., Meyer & Scherb. were collected, harvested, stored and treated as described previously (Scott and Oliver, 1994).

**Isolation of polysomes**

Polysomes were extracted from 0.5 g (Fwt prior to drying) of gametophytic tissue, unless stated otherwise, using either a high salt extraction buffer (200 mM sucrose, 200 mM Tris–Cl pH 8.5, 400 mM NaCl, 35 mM MgCl2, 5 mM DTT, 0.5 mM PMSF, 25 mM EGTA, 50 mg ml–1 cycloheximide, 0.5% (v/v) NP-40, and 10 mM ribonucleoside-vanadyl complex) or a low salt extraction buffer (200 mM sucrose, 200 mM Tris–HCl pH 8.5, 60 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 5 mM EGTA, 1% (v/v) NP-40, 0.5% (v/v) deoxycholate, 100 mg ml–1 heparin, 50 μg ml–1 cycloheximide, and 10 mM ribonucleoside-vanadyl complex). The 27,000 g supernatant was layered over a sucrose pad (1.5 M sucrose, 200 mM Tris–HCl pH 8.5, 60 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 100 mM Tris–Cl pH 8.5, 60 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 100 mg ml–1 heparin) and centrifuged at 100,000 g for 90 min in a 50TI ultracentrifuge rotor (Beckman Scientific, Fullerton, CA, USA). The polysomal pellet (100K g pellet) was solubilised in polysome-resuspension buffer (PRB) (10 mM Tris–Cl pH 7.5, 1 mM EDTA, 200 mM KCl).

**Sucrose and CsCl density gradient analysis**

For sucrose density gradient analysis of polysomes, the resuspended 100K g pellet was centrifuged through a sucrose cushion at 215,500 g for 75 min in a 5.0 ml, 10–50% sucrose density gradient (Oliver and Bewley, 1984a). For the generation of polyribosome profiles, the sucrose gradients were fractionated from the top with a density gradient fractionator (Beckman Scientific) for 75 min. After centrifugation, the CsCl gradients were collected from the top as 200 ml fractions, weighed to measure their density and monitored using an UA6 UV-detector (Isco) and the sucrose gradients were fractionated from the top with a density gradient fractionator (model 185; Isco, Lincoln, NE, USA). Absorbance at 254 nm was obtained using a UV–vis spectrophotometer (Beckman DU-800) and fractions were collected as 200 ml fractions. For CsCl density gradient analysis of polysomes, the polysomal pellet (100K g pellet) was solubilised in polysome-resuspension buffer (PRB) (200 mM sucrose, 200 mM Tris–Cl pH 8.5, 400 mM NaCl, 35 mM MgCl2, 5 mM DTT, 0.5 mM PMSF, 25 mM EGTA, 50 mg ml–1 cycloheximide, 0.5% (v/v) NP-40, and 10 mM ribonucleoside-vanadyl complex) or a low salt extraction buffer (200 mM sucrose, 200 mM Tris–HCl pH 8.5, 60 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 5 mM EGTA, 1% (v/v) NP-40, 0.5% (v/v) deoxycholate, 100 mg ml–1 heparin, 50 μg ml–1 cycloheximide, and 10 mM ribonucleoside-vanadyl complex). The 27 000 g supernatant was layered over a sucrose pad (1.5 M sucrose, 200 mM Tris–Cl pH 8.5, 60 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 100 mM Tris–Cl pH 8.5, 60 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 100 mg ml–1 heparin) and centrifuged at 100,000 g for 90 min in a 50TI ultracentrifuge rotor (Beckman Scientific, Fullerton, CA, USA). The polysomal pellet (100K g pellet) was solubilised in polysome-resuspension buffer (PRB) (10 mM Tris–Cl pH 7.5, 1 mM EDTA, 200 mM KCl).

**Quantitative RT–PCR**

Reverse transcriptase-PCR (RT–PCR) (Byrne et al., 1988) was used to evaluate the expression levels of a rehydrin transcript, Tr288 mRNA. To produce an internal mRNA standard (i.e. control template) which utilised the same PCR primers as wild-type Tr288 mRNA, a 66-bp Nrul fragment was removed from a cloned Tr288 cDNA. The Tr288-specific primers were 5′-CACTCATGAGC-CAGGTTTG-3′ (Upper) and 5′-TTTACAGATTGCCCCACTC-3′ (Lower). The lengths of the expected PCR products from cDNA produced by these primers is 307 bp (wild-type Tr288) and 241 bp (ΔNrul-Tr288). T3 DNA-dependent RNA-polymerase was used to produce ΔNrul-Tr288 mRNA from the cloned ΔNrul-Tr288 cDNA described above. Using total polysomal RNA, containing 20 ng of ΔNrul-Tr288 mRNA, a Tr288-specific primer, located near the 3 prime end of the mRNA, 5′-GGCAGAAAATGATGCAAATCTC-3′ (RT) was used to direct the RT-mediated production of single-stranded cDNA (total volume 50 ml). PCR was performed with 5 ml of the first-strand cDNA reaction. The reaction mixture was initially denatured at 95°C for 4 min. After the addition of Taq-polymerase (Promega, Madison, WI, USA), 25 cycles of amplification were performed at 95°C for 25 sec, 72°C for 30 sec and 95°C for 40 sec using a programmable thermal cycler (MJ Research, Inc., Watertown, MA, USA). The resulting PCR amplification products were resolved in 4% MetaPhor 1X TBE agarose gels (FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide and quantified against known DNA standards using a BioImage gel analyser (Millipore Corp., Bedford, MA, USA). The normalised readings are referred to as ‘relative units’. Pooled averages from three independent experiments were calculated. Standard curves were generated, using Tr288 and ΔNrul-Tr288 cDNA as template for PCR, to ensure that quantitative comparisons occur within a linear range of DNA amplification. Five μl aliquots were removed from sample reactions at 5 cycle intervals and quantified as described above. Twenty-five cycles is within the linear range of amplification for both Tr288 and ΔNrul-Tr288.

**Radiolabelling and UV-cross-linking of moss gametophytes**

Moss gametophytes were labelled with 100 μCi of Tran35S-label (ICN Pharmaceuticals, Irvine, CA, USA) and 100 μCi uridine [5,6-3H] (1.0 μCi ml–1) (NEN Inc., Boston, MA, USA) for 36 h, in autoclaved water, under continuous illumination (approximately 78 mmole m–2 sec–1) at 22°C (Oliver and Bewley, 1984b; Oliver and Bewley, 1984c). Dual-labelled samples, either 50 or 100 ml, were added to 5.0 ml ScintiSafe cocktail (Fisher Science, St Louis, MO, USA) and quantified using a TriCarb liquid scintillation analyser (Model 1500, Packard, Downers Grove, IL, USA) utilising standard default settings for dual-label recovery. Moss gametophytes were cross-linked in vivo by exposure to a hand-held UV source (254 nm; approximately 8 mW cm–2). Saturated, control gametophytes were continuously irradiated for 30 min. Desiccating gametophytes were removed from the drying chamber at 30, 60, 120, 240, and 360 min, irradiated for 5 min and returned to the drying chamber. Gametophytic tissue was flash-frozen and liquid
nitrogen and polysomes were isolated, as described previously, using the low salt extraction buffer.

Isolation of polysomal mRNP complexes and FPLC analysis

Polysomal mRNPs were isolated from the 100K g pellet from 4 g of gametophytic tissue by oligo-(dT) chromatography (Jain *et al.*, 1975), using DynaBeads oligo-(dT25) (Dyna, Inc., Lake Success, NY, USA), through two rounds of selection according to the manufacturers instructions. For FPLC analysis of the mRNP complexes, pooled samples from five separate oligo-(dT) purifications were analysed using a superose 6 HR10/30 column (Pharmacia, Inc., Piscataway, NJ, USA) in 100 mM Tris±Cl (pH 7.5). Samples were scanned at 280 nm to monitor the elution profile and molecular mass was determined, according to the manufacturers instructions, by establishing a standard curve using known globular protein (Pharmacia, Inc).

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