Transient Dissociation of Polyribosomes and Concurrent Recruitment of Calreticulin and Calmodulin Transcripts in Gravistimulated Maize Pulvini

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The dynamics of polyribosome abundance were studied in gravistimulated maize (Zea mays) stem pulvini. During the initial 15 min of gravistimulation, the amount of large polyribosomes transiently decreased. The transient decrease in polyribosome levels was accompanied by a transient decrease in polyribosome-associated mRNA. After 30 min of gravistimulation, the levels of polyribosomes and the amount of polyribosome-associated mRNA gradually increased over 24 h up to 3- to 4-fold of the initial value. Within 15 min of gravistimulation, total levels of transcripts coding for calreticulin and calmodulin were elevated 5-fold in maize pulvinus total RNA. Transcripts coding for calreticulin and calmodulin were recruited into polyribosomes within 15 min of gravistimulation. Over 4 h of gravistimulation, a gradual increase in the association of calreticulin and calmodulin transcripts with polyribosomes was seen predominantly in the lower one-half of the maize pulvinus; the association of transcripts for vacuolar invertase with polyribosomes did not change over this period. Our results suggest that within 15 min of gravistimulation, the translation of the majority of transcripts associated with polyribosomes decreased, resembling a general stress response. Recruitment of calreticulin and calmodulin transcripts into polyribosomes occurred predominantly in the lower pulvinus one-half during the first 4 h when the presentation time for gravistimulation in the maize pulvinus is not yet complete.

The vector of the gravitational force provides a constant cue for the direction of plant growth. Changes in the orientation of a plant relative to the gravity vector result in positive or negative gravitropic growth of roots and shoots, respectively. The gravity vector is thought to be perceived through changes in tensegrity or the pressure exerted by statoliths (Sack, 1991; Kaufman et al., 1995; Yoder et al., 2001) or by the entire protoplast (Staves, 1997). Sedimentation of statoliths in starch-containing cells can occur within seconds to minutes of gravistimulation (Sack, 1991; Kaufman et al., 1995; Yoder et al., 2001). A cascade of coordinated biochemical events subsequently amplifies and distributes the signal through a responsive tissue, resulting in the redistribution of auxin between upper and lower sides of the gravistimulated organ and initiating the bending response (for review, see Kaufman et al., 1995; Lomax et al., 1995; Sinclair and Trewavas, 1997; Chen et al., 1999; Rosen et al., 1999). Although the gravitropic response of plants has been the subject of intensive research, our understanding of the signaling processes linking perception of gravity to differential growth is still limited.

The stem pulvini of cereal grasses have previously been used as model systems for the investigation of gravitropic growth in a number of studies (Dayanan and Kaufman, 1984; Kaufman et al., 1987, 1995; Winter et al., 1997; Collings et al., 1998; Perera et al., 1999, 2001; Johannes et al., 2001). The pulvini are disc-shaped regions of the stem located immediately above the nodes; they contain starch granules and, importantly, are the exclusive site of gravitropic curvature in the stems of mature cereal grasses (Kaufman et al., 1987; Collings et al., 1998). The presentation time of the gravistimulus in the maize (Zea mays) pulvinus is between 2 and 4 h; gravistimulation for less than this duration will not result in bending if the plants are subsequently returned to a vertical orientation (Perera et al., 1999). The stem pulvini of 5- to 6-week-old maize stems respond to gravistimulation with differential elongation growth on the lower side after 8 h of continuous gravistimulation (Collings et al., 1998; Perera et al., 1999).

Perera et al. (2001) recently categorized biochemical events in the gravitropic response of cereal grass pulvini into three phases: early signaling during the presentation time (in maize, 0–4 h of gravistimulation), modulating the extent of differential growth (4–7 h), and metabolic changes driving differential elongation growth (>7 h). Rapid changes in the specific activity of phosphoinositide kinases (Perera et al., 1999), in the levels of the second messenger,
in ositol 1,4,5-trisphosphate (InsP₃; Perera et al., 1999), and in cytoplasmic pH (Johannes et al., 2001) have been reported to occur during the first phase. Sustained differential increases in the levels of InsP₃ and phosphoinositide kinases (Perera et al., 1999) and in the levels of auxin (J.C. Long, W. Zhao, A.M. Rashotte, S.C. Huber, and G.K. Muday, personal communication) have been described in the maize pulvinus system during the second phase (compare with Brock et al., 1991). Concurrent with the onset of gravitropic bending in phase three, transcript levels for the vacuolar acid invertase (ivr2) increase in the maize pulvinus after 8 h of gravistimulation (J.C. Long, W. Zhao, A.M. Rashotte, S.C. Huber, and G.K. Muday, personal communication). Changes in gene expression with gravistimulation have also been shown in other plant systems. These include the small auxin up-regulated genes (McClure and Guilfoyle, 1989; Li et al., 1991), IAA2 (Luschnig et al., 1998), and an auxin-induced K⁺ channel (Philippar et al., 1999).

Although an increase in the level of a transcript is indicative of an up-regulation of a particular gene product, increased transcript levels do not always result in increased synthesis of the respective protein (Browning, 1996; Hua et al., 2001). In addition to increased transcription, gravity-induced protein synthesis can be regulated at the level of translation by modulating the rates of initiation, elongation, and/or termination of ribosomes on a transcript. The dynamic assembly and disassembly of polyribosomes reflects the processes of initiation and termination, and the analysis of polyribosome profiles allows insight into global changes of the translational machinery and protein synthesis. Global changes in the rates of initiation or termination of ribosomes on mRNA may indicate stress- or stimulus-induced changes in protein synthesis (for review, see Bailey-Serres, 1999).

To understand posttranscriptional regulation of protein synthesis during the gravitropic response of maize stems, we have examined the dynamics of polyribosome formation and the recruitment of specific transcripts into polyribosomes over the first 4 h of gravistimulation. In this paper, we report a transient dissociation of polyribosomes in gravistimulated maize pulvinus that is correlated to an increase in recruitment of specific transcripts into polyribosomes. Transcript levels for calmodulin and calreticulin increased severalfold in total RNA from upper and lower pulvinus halves. Calreticulin and calmodulin are proteins potentially affecting cellular Ca²⁺ homeostasis and gravisensing (for review, see Sinclair and Trewavas, 1997). Transcripts coding for calreticulin and calmodulin were recruited into polyribosomes predominantly in the lower one-half of gravistimulated pulvini, suggesting recruitment of mRNA according to positional cues. The process occurs during the presentation time, when a commitment to differential growth has yet to be made.

RESULTS

The Abundance of Polyribosomes in Maize Pulvini Is Transiently Reduced during Gravistimulation

Polyribosomes were isolated from pulvinus tissue of vertical plants and from upper and lower pulvinus halves from maize plants gravistimulated for various time periods. Polyribosomes were purified and enriched by pelleting total extracts through 60% (w/v) Suc pads, and were then separated on 15% to 60% (w/v) Suc gradients, and polyribosome profiles were monitored by measuring the absorption at 254 nm as described previously (Davies and Abe, 1995). The identities of the 60S subunit, monosome, and polyribosomes bearing two, three, four, five, six, and more than six ribosomes are indicated in the profile obtained from control (vertical) tissue (Fig. 1A). Polyribosomes containing two to five ribosomes are considered small; large polyribosomes bear six or more ribosomes. Within the first 15 min of gravistimulation, a significant reduction in the abundance of large polyribosomes in the upper and lower pulvinus halves was observed (Fig. 1, B and C). However, by 30 min, the abundance of large polyribosomes had begun to increase in upper and lower halves (Fig. 1, D and E) so that by 60 min, the levels had returned to the initial values (Fig. 1, F and G). By 4 h (Fig. 1, H and I), a further increase in polyribosome content occurred, which continued over 24 h (Fig. 1, J and K).

To verify the transient decrease in polyribosomes during gravistimulation, the proportions of total polyribosomes (P) and large polyribosomes (LP) of the total ribosomal material (T) were calculated (as P/T% and LP/T%, respectively) and are shown separately for upper and lower halves of maize pulvini from plants gravistimulated for different durations (Fig. 2). The enrichment of polyribosomes on a Suc pad, as shown in Figure 1, although yielding more pronounced results, selects against the more slowly sedimenting particles such as subunits, monosomes, and small polyribosomes (Davies and Larkins, 1973). To include these components in the estimation of the total ribosomal material (T) and to correctly quantify shifts from one polysome class (e.g. large polysomes) to another (e.g. monosomes), profiles were obtained from total extracts directly layered onto Suc gradients (Davies and Abe, 1995). Polyribosome profiles obtained with the direct-layering method are shown in Figure 2A. Within 15 min of gravistimulation, P/T% decreased transiently by approximately 20% in both the upper and the lower pulvinus halves, recovered after about 30 min, and moderately increased thereafter (Fig. 2B). The pattern of changes in LP/T% (Fig. 2C) was similar to those in P/T% (Fig. 2B), with the exception that the transient decline in LP/T% at 15 min was approximately 25% in the lower one-half and
approximately 45% in the upper one-half. The transient decrease in P/T% indicates a shift from large polyribosomes to small polyribosomes, also consistent with a slow down in ribosome initiation. As with P/T% (Fig. 2B), by 30 min, the LP/T% ratio had recovered to the initial value and subsequently showed a moderate increase over 24 h (Fig. 2C).

Polysomal mRNA Dynamics during Gravistimulation

Figure 1. Transient decrease in polyribosomes during gravistimulation. Polyribosomes were enriched on 60% (w/v) Suc pads, separated on 15% to 60% (w/v) Suc gradients, and the UV absorption in the gradients was monitored. Polyribosome profiles shown are from maize P2-pulvinus tissue from vertical plants (A) and from upper (left; B, D, F, H, and J) and lower (right; C, E, G, I, and K) halves of plants gravistimulated for various durations, as indicated. The identities of the large ribosomal subunit (S), monosome (M), dimer (2), 3-mer (3), 4-mer (4), 5-mer (5), and 6-mer (6) are indicated in profile A. Small polyribosomes contain two to five ribosomes and large polyribosomes bear six or more. Polyribosomes bearing more than six ribosomes are indicated by the plus symbol. The cutoff between small and large polyribosomes is indicated by the dotted line. Two independent experiments gave consistent results.

The transient decrease in LP/T% is greater than that in P/T%, which indicates a shift from large polyribosomes to small polyribosomes, also consistent with a slow down in ribosome initiation. As with P/T% (Fig. 2B), by 30 min, the LP/T% ratio had recovered to the initial value and subsequently showed a moderate increase over 24 h (Fig. 2C).

Polyribosome-Associated mRNA Transiently Decreases within 15 Min and Subsequently Increases in Both Pulvinus Halves during Gravistimulation

The level of polyribosome-associated mRNA can be a measure of the recruitment of transcripts into polyribosomes. To determine if the amounts of polyribosome-associated mRNA changed during gravistimulation, profiles underlying the data presented in Figure 2 were subjected to detailed analysis of the amount of polyribosome-associated mRNA according to Davies and Larkins (1973). In brief, the polyribosome-associated mRNA in a given profile is quantified by dividing the area under each size class (e.g. 4 mer) by the number of ribosomes in that size class (i.e. four) and adding the values from all size classes for each profile. Monosomes are excluded from these calculations because unless they have been generated by RNase treatment, they rarely contain mRNA (Davies and Larkins, 1980). The relative amounts of polyribosome-associated mRNA in upper and lower halves of gravistimulated maize pulvini are shown in Figure 3. In upper (Fig. 3A) and lower (Fig. 3B) pulvinus halves, there was a gradual, approximately 3-fold increase in mRNA associated with polyribosomes within 24 h of gravistimulation.

It is interesting that within 15 min of gravistimulation, the amount of polyribosome-associated mRNA dropped by approximately 75% in upper and lower halves of stimulated pulvini. The amounts of polyribosome-associated mRNA were consistently decreased until about 60 min of gravistimulation, when the initial value was reestablished.

Transcript Levels for Calreticulin and Calmodulin Increase during Gravistimulation

To determine if the abundance of any transcripts changed in the maize pulvinus during gravistimulation, total RNA was isolated from upper and lower halves of maize pulvini harvested after various times of gravistimulation. Northern blots of total RNA probed with cDNAs representing calreticulin and calmodulin are shown in Figure 4A. The ratios of net band intensities for each transcript to those of the respective ribosomal RNA are given in Figure 4B. Increases in the levels of transcripts encoding calreticulin and calmodulin were evident after 15 min of gravistimulation, the earliest time point tested (Fig. 4A). Transcript levels for calreticulin increased about
5-fold within 15 min of gravistimulation and remained increased in the upper and lower pulvinus halves over the initial 2 h before reaching approximately 25 times the initial level in the lower pulvinus one-half after 4 h of gravistimulation (Fig. 4B, top). Transcript levels for calmodulin increased approximately 5-fold after 15 min of gravistimulation and remained elevated for at least 4 h, essentially equally in upper and lower pulvinus halves (Fig. 4B, bottom).

Calreticulin and Calmodulin Transcripts Are Rapidly Recruited into Polyribosomes during Gravistimulation

To determine whether the increases in calreticulin and calmodulin transcripts in total RNA (compare Fig. 4) were followed by the recruitment of these transcripts into polyribosomes, RNA was isolated from polyribosomes after separation on Suc density gradients and recording the UV absorption (profiles shown in Fig. 1). The presence and abundance of transcripts coding for calreticulin and calmodulin in the polysomal RNA was analyzed by non-saturating quantitative reverse transcriptase (RT)-PCR. This method was chosen over northern-blot analysis because of its greater sensitivity in detecting transcripts of low abundance in limiting amounts of polysomal RNA. Non-saturating conditions for quantitative amplification of invertase, calreticulin, and calmodulin fragments was carried out in 38, 31, and 33 cycles, respectively. The correlation between template input and amplifi-
cation of calmodulin fragments under these conditions is illustrated in Figure 5. Amplification was proportional to the template amount up to approximately 200 ng of total RNA input in all cases (data not shown).

Enhanced recruitment of transcripts for calreticulin and calmodulin into polyribosomes was evident in maize pulvini within 15 min of gravistimulation (Fig. 6). Transcript levels for acid invertase increase in the maize pulvinus after but not prior to the first 8 h of gravistimulation (J.C. Long, W. Zhao, A.M. Rashotte, S.C. Huber, and G.K. Muday, personal communication), and so the recruitment of invertase transcripts into polyribosomes was monitored as a control (Fig. 6A). Transcript levels for invertase in the RNA isolated from polyribosomes from pulvinus tissue harvested over a period of 4 h of gravistimulation did not change significantly (Fig. 6A), whereas in contrast, changes in transcript levels for calreticulin and calmodulin were observed in the same samples (Fig. 6, B and C). The ratios of net intensities of polysomal calreticulin and calmodulin transcripts to the net intensity of polysomal invertase transcript are given in Figure 6, B and C, respectively, as the fold increase over the ratio observed in vertical controls. Over a 4-h period of gravistimulation, transcript levels for calreticulin and calmodulin gradually increased in polyribosomes in the lower pulvinus one-half (Fig. 6, B and C). No such consistent increases were observed in the upper one-half over the period of treatment. Recruitment of calreticulin and calmodulin transcripts into polyribosomes during the first 4 h of gravistimulation was consistently greater in the lower pulvinus one-half than in the upper. It is interesting that the recruitment of calreticulin and calmodulin transcripts into polyribosomes occurred concomitantly with the transient decrease in polyribosomes (Figs. 1 and 2) and the decrease in polysome-associated mRNA (Fig. 3).

DISCUSSION

Between 1 and 24 h of gravistimulation, a gradual increase in the amount of polyribosomes and of polyribosome-associated mRNA was observed. These results suggest that during gravistimulation, protein synthesis in upper and lower pulvinus halves gradually increased. Because the non-perturbed pulvinus of a vertical mature maize stem is essentially quiescent, such a gradual increase after gravistimulation may reflect the initiation of the bending response and an overall increase in metabolic activity associated with the manifestation of differential growth.

It is interesting that preceding the gradual increase, within 15 min of gravistimulation, the abundance of polyribosomes transiently decreased in upper and lower halves of maize pulvini. At the same time, the amount of polyribosome-associated mRNA decreased by about 70% during the first 15 min of gravistimulation. The transient decrease in large polyribosomes within the first 15 min of gravistimulation can be explained by a transiently decreased rate of initiation and/or an increased rate of termination ("run-off") of ribosomes. With a decreased rate of initiation, large polyribosomes will progressively transform into smaller ones and into monosomes, resulting in reduced P/T% and LP/T%, respectively at around 15 min of gravistimulation.

At the onset of gravistimulation, large polyribosomes will primarily contain transcripts encoding housekeeping proteins translated in the unperturbed pulvinus and will not reflect gravity-induced protein synthesis. Our data suggest that upon gravistimulation, transcripts already in the process of translation
may not be as efficiently reinitiated into polyribosomes and may be released from polyribosomes. In a number of plant or plant cell suspension culture systems, the application of various stress conditions has been shown to impair translation of a majority of transcripts while selectively permitting the translation of others (Nover et al., 1989; Butler et al., 1990; Pittto et al., 1992; Reinbothe et al., 1993; Fennoy and Bailey-Serres, 1995; Gallie et al., 1995; Fennoy et al., 1998; for review, see Bailey-Serres, 1999). Anoxia globally impairs translation while allowing the selective translation of anaerobic polypeptides in potato (*Solanum tuberosum*; Butler et al., 1990) and in maize seedlings (Fennoy and Bailey-Serres, 1995; Gallie et al., 1995; Fennoy et al., 1998; for review, see Bailey-Serres, 1999). Anoxia globally impairs translation while allowing the selective translation of anaerobic polypeptides in potato (*Solanum tuberosum*; Butler et al., 1990) and in maize seedlings (Fennoy and Bailey-Serres, 1995; Fennoy et al., 1998; Manjunath et al., 2001). Heat shock results in a global impairment of translation, whereas heat shock proteins are selectively translated (Nover et al., 1989; Pittto et al., 1992; Gallie et al., 1995). Application of jasmonic acid specifically impairs the translation of certain transcripts, whereas the translation of others is selectively increased (Reinbothe et al., 1993). The main cause for globally decreased translation in plants has been suggested to be a decreased rate of ribosome initiation (Bailey-Serres, 1999). The transient decrease in the abundance of polyribosomes and in polyribosome-associated mRNA in the gravistimulated maize pulvinus reported here resembles a global decrease in translation typical for plant stress responses mentioned above.

In analogy to the effects of various stresses on translation, we asked whether concurrent with the globally decreased rate of translation in gravistimulated maize pulvinus, the recruitment of specific transcripts would be favored. To our knowledge, selective recruitment of mRNA into polyribosomes during the gravitropic response of plants has not been previously reported. Northern-blot experiments with total RNA from gravistimulated maize pulvinus showed that within 15 min of gravistimulation, the levels of transcripts encoding calreticulin and calmodulin increased approximately 5-fold. To our knowledge, the gravity-induced increase in calreticulin transcript is a
The increased association of calreticulin and calmodulin transcripts with polyribosomes in the lower pulvinus one-half might imply increased synthesis of calreticulin and calmodulin protein. The timing of this putative increased translation of calreticulin and calmodulin suggests a role for these proteins in gravipositioning rather than in driving elongation growth because after 15 to 30 min of gravistimulation, i.e. during the phase of early signaling, a new growth axis and a commitment to gravitropic growth has yet to be established (Perera et al., 1999).

It has been hypothesized that rapid, localized Ca2+ fluxes represent a very early step in the gravitropic signal transduction cascade that could be mediated through pressure or turgor changes exerted by statoliths or the whole protoplast on the cytoskeleton, activating mechanosensitive Ca2+ channels (Ding and Pickard, 1993; Yoder et al., 2001). However, as of now there is no positive report of such rapid Ca2+ changes during plant gravitropism (Legue et al., 1997). However, long-term alterations in the levels of cytosolic Ca2+ during gravitropism have been monitored in a number of plant systems (for review, see Lee et al., 1983; Gehring et al., 1990; Bjorkman and Cleland, 1991; Belyavskaya, 1996; Sinclair and Trewavas, 1999). Indirect evidence such as a gradual increase in the Ca2+-mobilizing phosphoinositide InsP3 in gravistimulated maize pulvini (Perera et al., 1999) is consistent with a role for Ca2+ during the phase of modulating the extent of differential growth in maize gravitropism. Calreticulin is a Golgi- and endoplasmic reticulum (ER)-localized protein that can act as a store for Ca2+ ions (Dresselhaus et al., 1996; Fagny et al., 2000). Overexpression of maize calreticulin has recently been shown to perturb Ca2+ homeostasis in transgenic tobacco cells (Persson et al., 2001) and to increase the survivability of transgenic Arabidopsis seedlings grown on low-Ca2+ media (Persson et al., 2001). In addition, calreticulin can function as a chaperone and may be necessary for correct protein folding in the ER (Borisjuk et al., 1998). Calmodulin is an important player in Ca2+ signal transduction (Zielinski, 1998) and is involved in sensing intracellular Ca2+ levels. An increase in calreticulin and calmodulin synthesis in the lower one-half of gravistimulated maize pulvini may indicate a gradual alteration in cellular Ca2+ homeostasis, which may be reflected by changes in Ca2+ storage capacity through calreticulin in the ER, gradually increasing levels of InsP3 and Ca2+, and by altered Ca2+ sensitivity, through increased levels of calmodulin.

In summary, the global nature of the transient dissociation of polyribosomes implies that after 15 min of gravistimulation there is a transient decrease in translation on the majority of transcripts associated with polyribosomes. A transient dissociation of the protein-synthetic machinery early during the presentation time may indicate a shift in physiological activity of the pulvinus tissue according to a general novel finding. The expression of calreticulin has previously been shown to increase during the early stages of somatic embryogenesis in tobacco (Nicotiana plumbaginifolia) and to be influenced by exogenous auxin in tobacco cell cultures (Borisjuk et al., 1998). Up-regulation of calmodulin transcripts in Arabidopsis roots gravistimulated for 30 min has previously been reported; however, it is not clear whether the increase in calmodulin transcript occurred within or prior to the presentation time (Sinclair et al., 1996). Transcripts for calreticulin and calmodulin were rapidly recruited into polyribosomes in upper and lower halves of gravistimulated maize pulvini. In the upper one-half, increases and decreases in the levels of polyribosome-associated calreticulin and calmodulin transcripts alternated over the first 4 h of stimulation, whereas there was a gradual increase in the levels of calreticulin and calmodulin transcripts associated with polyribosomes in the lower pulvinus one-half. In contrast, the abundance of transcripts for the vacuolar acid invertase ivr2 did not change in polyribosomes from upper or lower pulvinus halves over the initial 4 h of gravistimulation. The asymmetry of recruitment of calreticulin and calmodulin transcripts into polyribosomes is suggestive of the preferential translation of these mRNAs not only in contrast to the global decrease in translation, but also according to positional cues in the pulvinus tissue.
stress signal, which may induce further perception and signaling mechanisms. During this period, calreticulin and calmodulin transcripts are recruited into polyribosomes predominantly on the lower one-half of the pulvinus. Increased synthesis of calreticulin and calmodulin after gravistimulation would affect cellular Ca\(^{2+}\)/H\(^{+}\) homeostasis and may reflect a changed capacity and sensitivity for Ca\(^{2+}\)/H\(^{+}\) signals during the early steps of the gravity response in maize.

**MATERIAL AND METHODS**

**Plant Material**

Maize (*Zea mays* cv Pioneer 3183) plants were grown in soil in 20-cm pots (four plants per pot) under natural lighting in a greenhouse. Plants were fertilized with a modified Hoagland solution every 2nd d. The most graviresponsive P2 pulvinus (the first pulvinus above the soil line) of 6-week-old maize plants was chosen for our analyses (compare with Collings et al., 1998). During growth, stimulation, and harvesting, care was taken to minimize handling and movement of the plants. Plants were gravistimulated for the indicated times by placing the pots horizontally. Pulvini of gravistimulated plants were dissected into upper and lower halves while maintaining a horizontal orientation. Control plants were kept vertical, and control pulvini were cut in halves on a random plane. Dissected pulvinus tissue was frozen immediately in liquid nitrogen. Tissue samples were stored at −80°C.

**Isolation of Total RNA**

Pulvinus tissue from upper and lower halves from one or two pulvini or from vertical pulvinus tissue (approximately 0.2 g fresh weight) was ground to a fine powder in liquid nitrogen. Total RNA was isolated using the plant RNeasy kit (QIAGEN, Valencia, CA) according to manufacturer’s instructions. The recovery of RNA was quanti-
from the top of the gradient by displacement with 80% (w/v) Suc in water. The absorption of the gradient at 254 nm was continuously monitored using a UA-6 UV-Detector (ISCO, Lincoln, NE) linked to a Type 11 Optical Unit (Isco).

Quantification of Protein Content

Protein concentrations were estimated by using the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Isolation of RNA from Polyribosomes

RNA was isolated from polyribosome fractions by acid phenol extraction by adding phenol:chloroform (5:1 [v/v], pH 4.5, Ambion), washing the aqueous phase with chloroform:isoamyl alcohol (24:1 [v/v]), and by precipitating RNA with ethanol containing 0.3 M ammonium acetate at −20°C overnight.

Reverse Transcription and Quantitative, Non-Saturating PCR

Reverse transcription was carried out under uniform conditions using 60 ng of polysomal RNA and 3 ng of RT primer: 5′-TTCATGCCGCGCGGGGAGCATGTGAGCTCGGTG-3′ (sense), 5′-CGTGGTTGATGACG-3′ (antisense); calreticulin (accession no. AF190454), and calmodulin (accession no. X74490) radiolabeled by random priming with α[32P]dCTP. Blots were washed twice at room temperature in 2× SSPE/0.2% (w/v) SDS, followed by washes in 1× SSPE/0.1% (w/v) SDS at room temperature and hybridization temperature. The final washes were in 0.1× SSPE/0.1% (w/v) SDS at 44°C and 55°C. Hybridization was visualized by autoradiography. Autoradiographs and blots stained with methylene blue were sized using a DC120 digital camera (Eastman Kodak). Band intensities were quantified using Digital Science 1D version 2.0.2 imaging software (Eastman Kodak).

Isolation of Polyribosomes

All equipment was pretreated with RNase Zap (Ambion, Austin, TX) and prechilled. Pulvinus tissue (approximately 2 mL) was resuspended in 400 μL of water, and loaded onto 4 mL. 15% to 60% (w/v) Suc gradients in buffer B. Gradients were centrifuged at 4°C and 3°C; and calmodulin, respectively. Amplification was proportional to an amount of RNA in-
ul, 29 cycles, annealing at 55°C. Figure 5 illustrates template-dependent amplification of the three fragments up to 125 ng of total RNA input under the specified conditions. No specific PCR products were obtained with RNA starting material exceeding 250 ng (calmodulin) or 300 ng (calreticulin and invertase). DNA fragments were analyzed by gel electrophoresis on 1.2% or 1.4% (w/v) agarose gels and were visualized with ethidium bromide. Amplification of polysomal cDNA with primers specific for ivr2, calreticulin, or calmodulin sequences (see above) yielded PCR products of sizes predicted from the respective gene sequences (approximately 580, 380, and 330 bp, respectively). Agarose gels were imaged under UV illumination using a DC120 digital camera (Eastman Kodak). Band intensities were quantified using Digital Science 1D version 2.0.2 imaging software (Eastman Kodak).

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LITERATURE CITED


