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Stress-Induced Translational Control in Potato Tubers May Be Mediated by Polysome-Associated Proteins

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Potato tubers exhibit distinct responses to wounding and hypoxia that include selective translation of stress-induced mRNAs. Newly synthesized wound-response mRNAs are bound to polysomes, whereas preexisting mRNAs are displaced and degraded. mRNAs that are induced and translated during hypoxic conditions are bound to ribosomes as expected. However, preexisting wound-response mRNAs whose translation is inhibited during hypoxia remain bound to polysomes, indicating that there are at least two distinct mechanisms by which translation is regulated in response to stress conditions. A 32-kD phosphoprotein is associated with polyribosomes from wounded tubers. This protein remains polysome bound as long as wound-response mRNAs are present, even during hypoxia when these mRNAs are no longer translated. However, association of the 32-kD protein with polysomes is not elicited by hypoxic stress alone. The kinase that phosphorylates this protein is active only for the first 24 hr after wounding and is not active during periods of hypoxia. This protein may mediate recognition of the wound-response mRNAs by ribosomes.

INTRODUCTION

The responses of potato tubers to the two environmental stresses, wounding and hypoxia, mediate resistance and susceptibility to bacterial disease (for review, see Davis et al., 1990). The tuber wound response elicits synthesis of a number of gene products such as phenylalanine ammonia-lyase (PAL) (Vayda and Schaeffer, 1988; Bevan et al., 1989; Butler et al., 1990), the glycine-rich and hydroxyproline-rich cell wall proteins (Butler et al., 1990; Rumeau et al., 1990), peroxidase (Roberts et al., 1988), DHAP synthetase (Dyer et al., 1989), histone H4 (Butler et al., 1990), *wun1* (Logemann et al., 1989), and *win2* (Stanford et al., 1989, 1990). Many of these genes are induced in other plant species upon wounding as well (Bolwell et al., 1985; Chen and Varner, 1985; Cramer et al., 1985; Showalter et al., 1985; Davis and Hahlbrock, 1987; Lawton and Lamb, 1987; Bevan et al., 1989). Several of these gene products are involved in the synthesis of cell walls and the processes of lignification, suberization, or cell division. These activities contribute to the formation of a wound periderm that may act as a physical barrier limiting pathogen infection (Dean and Kolattukudy, 1976; Barkhausen, 1978; Kolattukudy, 1981; Davis et al., 1990). Hypoxic stress elicits the synthesis of a set of polypeptides distinct from that of the wound response (Vayda and Schaeffer, 1988). Among the genes induced by hypoxia are alcohol dehydrogenase (ADH) and aldolase (ALD) (Vayda and Schaeffer, 1988; Butler et al., 1990). These and other genes whose products are involved in fermentative metabolism are induced in a variety of plants during hypoxic conditions (Hake et al., 1985; Good and Crosby, 1989; Kelley, 1989; Ricard et al., 1989, 1991; Xie and Wu, 1989). The activities of these gene products may enhance survival in low oxygen conditions.

In addition to the induction of specific genes, both stress responses rapidly inhibit translation of the mRNA species present before onset of the stress condition (Butler et al., 1990). For example, mRNAs that remain present after wounding or hypoxic stress are translated efficiently in vitro (Butler et al., 1990) but are not translated in vivo during either of the stress conditions (Vayda and Schaeffer, 1988). By contrast, two abundant mRNAs present before wounding, patatin and proteinase inhibitor II (PI-II), are specifically and rapidly degraded upon wounding (Logemann et al., 1988; Butler et al., 1990). The wound-induced mRNAs that are the predominant messages translated in vivo upon wounding (Vayda and Schaeffer, 1988) comprise only a small fraction of the total tuber mRNA and are barely detectable upon translation in vitro of total tuber RNA (Butler et al., 1990). Thus, some mechanism must exist for the selective translation of the wound-response messages.

By contrast, the wound-induced mRNAs are not translated in vivo within 30 min of transfer to hypoxic conditions, although their steady-state levels remain constant for up

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to 12 hr and significant amounts (>50%) remain even after 24 hr of hypoxia (Butler et al., 1990). mRNAs of hypoxia-induced genes such as ADH and ALD accumulate to a high level between 12 and 24 hr of hypoxia and are translated (Butler et al., 1990). Thus, a second mechanism must exist for the selective translation of hypoxia-inducible mRNA in the presence of wound-response mRNA.

The objective of this study was to provide insight into the mechanisms by which tubers selectively translate distinct classes of stress-induced mRNAs and effect the inhibition of other mRNAs. The studies of Berry and Klessig provide examples by which translation in plants is regulated in two distinct ways: by preventing initiation on specific mRNAs (Berry et al., 1990) and by blocking elongation (Berry et al., 1988). Ribulose-1,5-bisphosphate carboxylase small subunit mRNA (SSU) is not bound to polysomes of dark-grown amaranth cotyledons, but becomes associated and is translated upon transfer to light (Berry et al., 1990). By contrast, SSU mRNA remains bound to polysomes, although no longer translated, when seedlings are transferred to the dark (Berry et al., 1988). These studies indicate that some factors must be present that are either necessary for, or inhibitory to, translation during specific conditions. Our results indicate that wounding inhibits translation of the mRNAs encoding the major tuber proteins by causing them to dissociate immediately from polysome complexes. In contrast, hypoxia arrests translation of wound-response and other preexisting mRNAs, although these mRNAs remain bound to polysomes. Thus, both inhibition of reinitiation and elongation appear to be used to regulate translation in response to different environmental stress conditions. Furthermore, we have identified a protein that may mediate recognition of wound-induced mRNAs by ribosomes: a 32-kD phosphoprotein that is associated with polysomes when wound-response mRNA is bound.

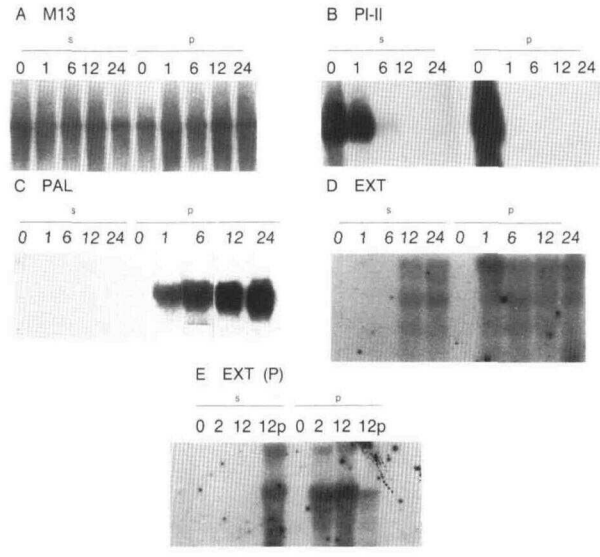

Figure 1. mRNAs Associated with Polysomes of Nonwounded and Wounded Tubers.

RNA isolated from the supernatant (s) and polysome (p) fractions of samples isolated from nonwounded (0) tubers or tubers wounded for the time (in hours) indicated were resolved by RNA gel blot hybridization to the ^{32}P-labeled random primer probes indicated. Forty nanograms of single-stranded M13 DNA was added to the supernatant and polysome fractions before isolation of nucleic acid to allow loading of RNA reflecting the original proportions in the two fractions.

(A) M13 probe.
(B) Proteinase inhibitor II probe.
(C) PAL probe.
(D) Extensin probe.
(E) Extensin probe of sample set including puromycin-treated (12p) material.

RESULTS

RNA Associated with Polysomes from Wounded Tubers

Figure 1 shows the association of specific messages with polysomes isolated from nonwounded and wounded tubers. During isolation, equal amounts of single-stranded M13 DNA were added to the supernatant and pellet fractions. This normalization, shown in Figure 1A, allowed the most accurate loading of RNAs that reflected actual proportions present in the supernatant and polysome-associated fractions, despite the widely disparate volume, sucrose content, and RNA composition of the two fractions. In Figure 1B, it is clear that PI-II RNA, a marker of the abundant, sucrose-inducible, tuberization-specific transcripts, was associated with the polysome fraction before wounding. Only approximately 20% of the total PI-II RNA was found in the "free RNA" or supernatant fraction. However, within 1 hr of wounding, no PI-II RNA was found in the polysome fraction. PI-II RNA was apparently displaced into the free RNA pool where it was rapidly degraded (Figure 1B). Exactly the same result was obtained when polysomal RNA was probed for patatin, another abundant, sucrose-inducible, tuberization-specific message (data not shown). Thus, the major tuber mRNAs were polysome bound in nonstressed tubers and were displaced from ribosomes by wounding.

In contrast, Figures 1C and 1D show that wound-induced messages for PAL and extensin (EXT) became associated with ribosomes soon after wounding, as expected. A 2.7-kb RNA species that hybridized to radiolabeled PAL DNA appeared within 1 hr after wounding and was entirely associated with the polysome fraction. High steady-state levels of this RNA remained in the polysome fraction for at least 24 hr after wounding (Figure 1C). The

several characteristic RNAs that hybridized to radiolabeled EXT DNA (Butler et al., 1990; Rumeau et al., 1990) also remained in the polysome fraction for at least 24 hr after wounding (Figure 1D). It is not known why significant amounts of the EXT species consistently accumulated in the supernatant fraction as well. Only occasionally were minor amounts of PAL or other wound-induced RNAs detected in the supernatant fraction of 24-hr wounded tubers (data not shown). These free species may either reflect turnover of the wound-response messages or indicate that more wound-response RNA was present at these times than could be accommodated by ribosome complexes. In either case, RNA species representative of the wound response were predominantly found in the polysome fraction during the peak period of wound-response protein synthesis.

The wound-response RNAs could be displaced from the polysome fraction into the free RNA supernatant by incubation with 5 μg/mL puromycin (Figure 1E). This result suggested that these species were ribosome-associated and did not pellet simply because they were in a complex with some other material that exhibited a high sedimentation coefficient. Material present in the polysome fraction exhibited values of 40 S or greater, as estimated by the sedimentation of radiolabeled RNA markers of known length. Under these conditions, some 9.5-kb RNA molecules pelleted, whereas the majority of the 9.5-kb molecules and all RNA of shorter length remained in the supernatant fraction (data not shown). Further evidence that wound-response RNAs were polysome associated is presented in Figure 2. PAL RNA was found associated with oligomeric ribosomes when the polysome fraction was resolved further by sedimentation through a 5% to 20% sucrose gradient. These data, taken together, suggested that the hybridizing RNAs were sedimenting with the pellet fraction by virtue of their association with ribosome complexes.

Evidence presented in Figure 3 indicates that polysomes in the pellet fraction were competent for runoff translation in vitro using rabbit reticulocyte lysates. Purified RNA from nonwounded and wounded tubers was efficiently translated in vitro and produced nearly identical products, as has been noted previously (Butler et al., 1990). However, the addition of 200 μM 7-methylguanosine (m^7 GMP) and 25 μg/mL chloramphenicol (Berry et al., 1990) effectively inhibited translation of deproteinized RNA by at least 90% (Figure 3), presumably by preventing initiation. However, the synthesis of translation products by polysomes isolated from nonwounded or 16-hr wounded tubers was unaffected by the presence of the initiation inhibitor m^7 GMP (Figure 3). Under these conditions, factors present in the reticulocyte lysate allowed completion of translation by tuber polysomes initiated in vivo, but reinitiation of translation by reticulocyte ribosomes did not occur. Furthermore, completion of translation occurred as efficiently at temperatures favoring tuber ribosome activity (20°C) as

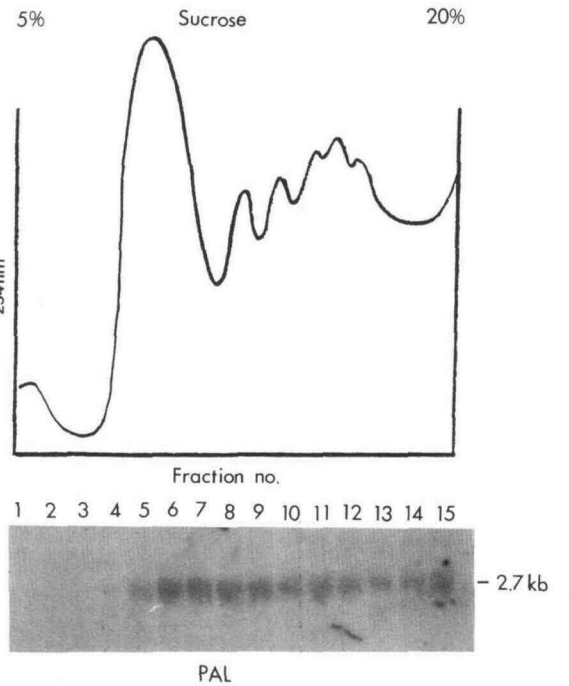

Figure 2. PAL mRNA Associated with Fractionated Polysomes.

Pelleted polysomal material was further resolved by centrifugation through a 5% to 20% linear sucrose gradient. Fractions were collected using an Isco density gradient fractionator, model 640 (Isco, Lincoln, NE). Deproteinized RNA was resolved through a 1.5% agarose gel, transferred to nylon, and probed by hybridization to ^{32}P-labeled PAL cDNA.

at higher temperatures (37°C) favoring rabbit reticulocyte ribosome activity.

The most abundant translation products generated by 16-hr wounded polysomes exhibited mobilities similar to those of PAL (78 kD), DHAP synthase (50 kD), and the other major proteins labeled in vivo after wounding (Vayda and Schaeffer, 1988; Butler et al., 1990). However, these polypeptides were not the most abundant products of the translation of deproteinized RNA (Figure 3), as observed previously (Butler et al., 1990). The major runoff translation products generated in vitro by polysomes isolated from nonwounded tubers exhibited mobilities of 40 kD, 24 kD, 20 kD, <10 kD, and 100 kD, consistent with the major proteins of mature tubers, specifically patatin, and the proteinase inhibitors p22, PI-II, and PI-I. However, polysomes isolated from nonwounded tubers exhibited a poor ability to produce translation products by runoff in vitro. Polysomes from nonwounded tubers exhibited fivefold to 20-fold less incorporation of ^{35}S-methionine than the same amount (10 μg) of polysomes isolated from 16-hr wounded tubers. This was unexpected considering the abundance

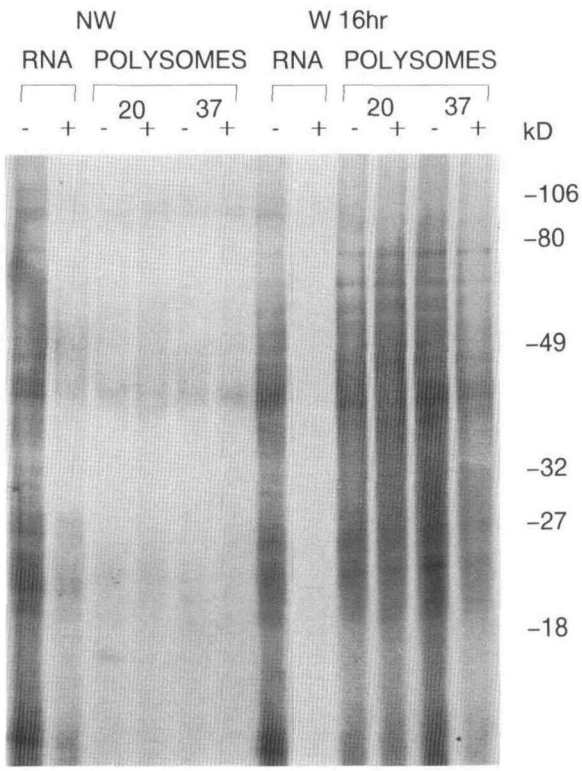

Figure 3. Translational Runoff of Polysomes in Vitro.

Deproteinized RNA or polysomes from nonwounded (NW) or 16-hr wounded (W 16hr) tubers were incubated with rabbit reticulocyte lysates in the absence (–) or presence (+) of the initiation inhibitor m^7 GMP. RNA was incubated at 37°C for 1 hr; polysomes were incubated for 1 hr at 20°C or 37°C, as indicated. ^{35}S-methionine-labeled translation products were resolved by SDS-PAGE and fluorography. Sizes indicated are relative to migration of known markers.

of mRNAs associated with polysomes of nonwounded tubers apparent in Figure 1.

A 32-kD Phosphoprotein Is Associated with Polysomes Isolated from Wounded Tubers

To understand the basis for these differences between polysomes from nonwounded and wounded tubers, the polypeptides associated with each fraction were analyzed by SDS-PAGE. The protein composition of wounded and nonwounded polysomes appeared similar by this analysis, with the exception of a prominent 32-kD protein, possibly a doublet, that was evident in the polysome fractions from wounded tubers, as shown in Figures 4, 5, and 6. The lower, 30-kD component was observed in both nonwounded and wounded polysome fractions. In contrast, the 32-kD protein was the most prominent species of the polysome fraction from wounded tubers (Figure 4A). Although the 32-kD protein was not evident in the supernatant fractions of Figure 4C, the large volume, high sucrose, and high protein content of the supernatant did not allow detection of small amounts of 32-kD protein that may have been present in this fraction. Thus, it is not clear whether this protein is distributed between both fractions or is present in the supernatant before wounding.

The 32-kD protein was phosphorylated in vivo as is evident in Figure 4B by the incorporation of ^{32}P-orthophosphate supplied to whole tubers. Indeed, the 32-kD protein was the major phosphorylated species associated with the polysome fraction of wounded tubers. Either the activity was activated upon wounding or the 32-kD protein substrate was not present before wounding because labeling of a 32-kD species was not apparent in nonwounded tubers by incorporation of ^{32}P-orthophosphate through

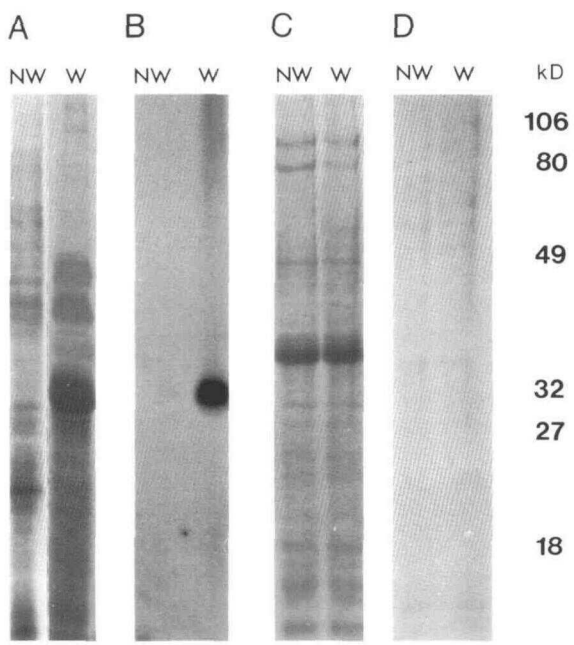

Figure 4. Polysome-Associated Phosphoproteins of Nonwounded and Wounded Tubers.

^{32}P-orthophosphate (0.5 mCi) was presented to nicked eyebuds of nonwounded tubers (NW) or tubers wounded 1 cm from the eyebud 2 hr before harvest (W). Label was incubated with tissue for 1 hr in each case. Polypeptides were resolved by SDS-PAGE. Sizes indicated are relative to markers of known length.

(A) Polypeptides of the polysome fractions visualized by staining with Coomassie blue.

(B) ^{32}P-labeled phosphoproteins of the polysome fractions detected by autoradiography of gel shown in **(A)**.

(C) Coomassie blue-stained polypeptides of the supernatant fractions.

(D) ^{32}P-labeled phosphoproteins of the supernatant fractions detected by autoradiography of the gel shown in **(C)**.

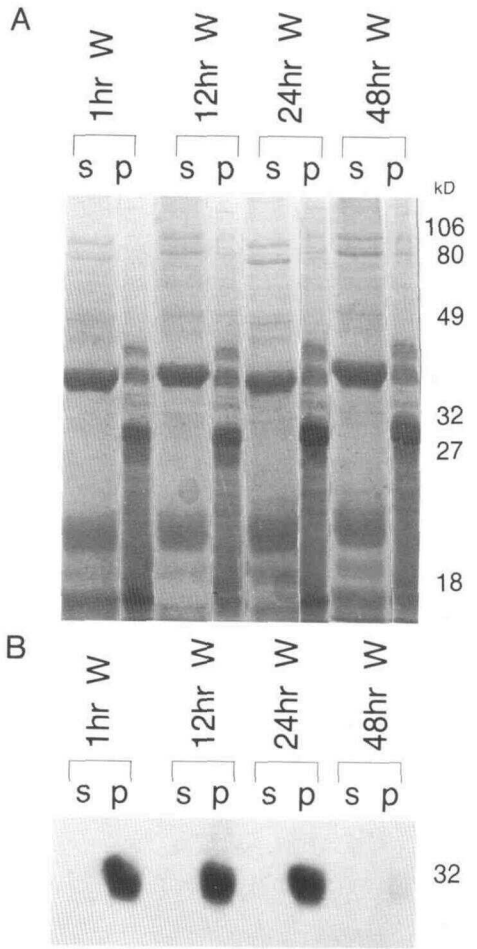

Figure 5. Polypeptides Associated with Polysomes Isolated from Wounded Tubers.

(A) Polypeptides of the supernatant (s) and polysome (p) fractions of tubers wounded (W) for the times indicated were resolved by SDS-PAGE and staining with Coomassie blue. Equal volumes (100 μL) of samples were loaded.

(B) Autoradiography of the same samples shown in **(A)**. ^{32}P-orthophosphate (0.25 mCi) was added to the wound sites 30 min before harvest. Sizes indicated are relative to migration of known markers.

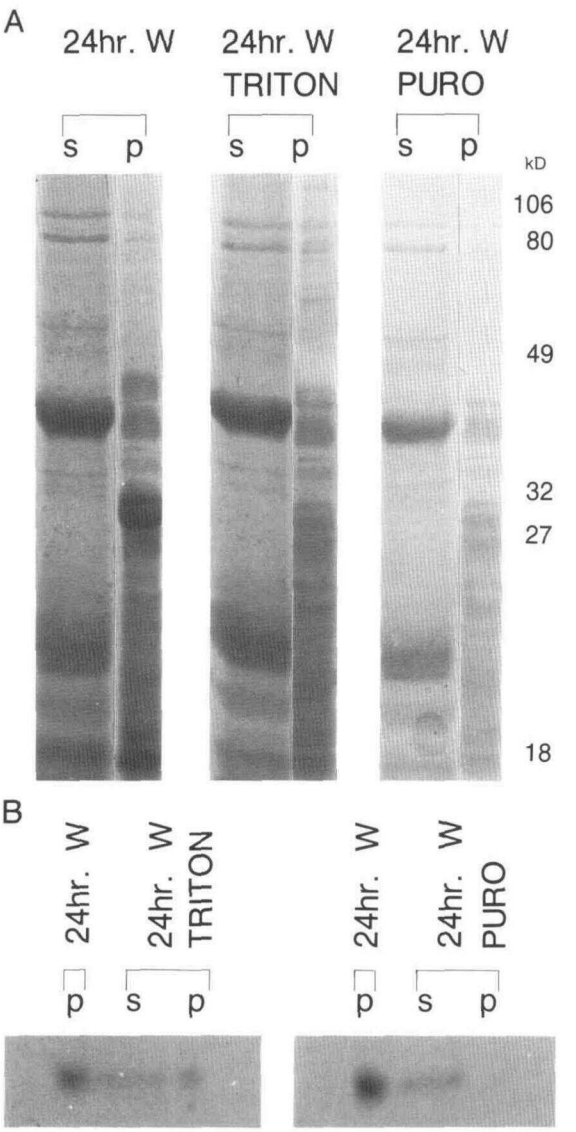

Figure 6. Dissociation of the 32-kD Phosphoprotein from Polysomes by Treatment with Triton or Puromycin.

Polysomes were isolated from a tuber wounded for 24 hr (24 hr W). ^{32}P-orthophosphate (0.25 mCi) was incubated in wounds for 30 min before harvest. Before centrifugation, one-third of the sample was treated with 0.1% Triton X-100 (TRITON) and one-third was incubated with 5 μg/mL puromycin (PURO) for 15 min at 37°C. Polypeptides in equal volumes of the supernatant (s) and polysome (p) fractions were resolved by SDS-PAGE. Sizes indicated are relative to migration of known markers.

(A) Visualization of polypeptides by staining with Coomassie blue.

(B) Autoradiographic detection of the 32-kD phosphoprotein in equal volumes of the supernatant and polysome fractions of Triton X-100- and puromycin-treated samples, compared with the polysomal fraction of the untreated 24-hr wounded sample.

eyebuds (Figure 4B). By contrast, tuber tissue wounded 0.5 cm from the eyebud, where label was presented, did exhibit substantial amounts of phosphorylated 32-kD protein (Figure 4B). In Figure 4D it is clear that nonwounded tubers were competent to incorporate the ^{32}P-orthophosphate because proteins in the supernatant fractions of nonwounded and wounded tubers were labeled to the same extent.

The 32-kD protein was found associated with polysome complexes very soon after wounding, in some trials within

15 min (data not shown), but typically within 1 hr after wounding. As shown in Figure 5A, this protein remained associated for up to 48 hr after wounding, paralleling the presence of known wound-response RNAs (Butler et al., 1990). The presumed nucleotide triphosphate-dependent protein kinase was active as early after wounding as the 32-kD protein was detected in association with polysomes (Figure 5B). The activity that incorporated ^{32}P label into the 32-kD protein persisted for 24 hr after wounding (Figure 5B), diminished by 32 hr after wounding (data not shown), and was not detected 48 hr after wounding (Figure 5B). However, the 32-kD polypeptide remained polysome bound 48 hr after wounding, as evidenced by the staining in Figure 5A. This bound protein was apparently in a phosphorylated form because ^{32}P-labeled 32-kD protein pulse-labeled 12 hr after wounding remained polysome associated 48 hr after wounding and 35 hr after chase with 10 mM unlabeled sodium phosphate, pH 7.0 (data not shown). The observed diminution of the putative kinase activity by 48 hr after wounding correlated with the passing of the wound response and the return to basal tuber metabolism, which has been reported to occur by 72 hr after wounding (Logemann et al., 1988).

The phosphorylated 32-kD species is unlikely to be ribosomal protein S6, previously reported to be phosphorylated in plants before application of stress conditions (Scharf and Nover, 1982; Bailey-Serres and Freeling, 1990), because both the 32-kD protein (Figure 6A) and the phosphorylated species (Figure 6B) were displaced by incubation with 5 μg/mL puromycin. In Figure 6B, the ^{32}P signal of the displaced phosphoprotein in the supernatant fraction was similar to that remaining in an equal volume sample of the polysome fraction. The total volume of the supernatant (13 mL) was more than 20-fold that of the polysome fraction (0.5 mL). Thus, the equal intensity of this species in both fractions indicated that >95% was present in the supernatant and <5% remained polysome associated after puromycin treatment. This was confirmed by the diminished presence of the 32-kD protein in stained gels (Figure 6A). Neither such dissociation from polysomes nor the observed molar excess of the 32-kD protein relative to other ribosome-associated proteins would be expected of an integral ribosomal protein such as S6. Furthermore, it was clear from the data shown in Figure 6 that the 32-kD protein could be stripped from the polysome fraction by a gentle wash with 0.1% Triton X-100. Thus, the 32-kD protein is most likely a peripherally associated ribosomal protein or perhaps a protein bound to wound-response RNAs.

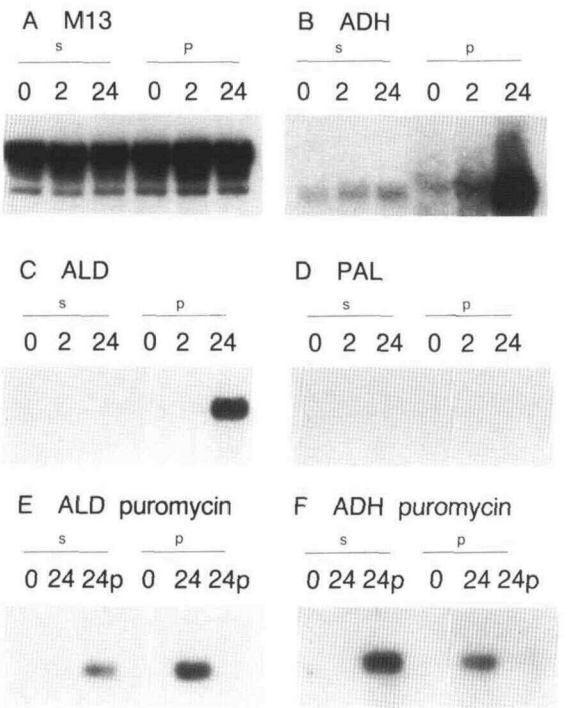

Figure 7. mRNAs Associated with Polysomes from Hypoxic Tubers.

RNA extracted from the supernatant (s) and polysome (p) fractions of aerobic tubers (0) and tubers hypoxic for 2 hr or 24 hr were resolved by electrophoresis through 1.5% agarose gels and detected by hybridization to the ^{32}P-labeled random primer probes indicated.

(A) Normalization control by hybridization to ^{32}P-M13 DNA.
(B) ADH probe.
(C) ALD probe.
(D) PAL probe.
(E) ALD probe of sample set including puromycin-treated (24p) material.
(F) ADH probe of sample set including puromycin-treated (24p) material.

RNA Associated with Polysomes from Hypoxic Tubers

Figures 7B and 7C show that the hypoxia-induced RNA, represented by ADH and ALD, were almost entirely associated with the polysome fraction of nonwounded hypoxic tubers. PAL RNA was not induced by hypoxic stress alone (Butler et al., 1990), and, accordingly, the labeled PAL probe did not detect any hybridizable material in either the polysome or supernatant fractions of hypoxic tubers (Figure 7D). No change was detected in the distribution of patatin and PI-II RNA between polysome and supernatant fractions when nonwounded tubers were incubated for up to 24 hr in hypoxic conditions: the species encoding these two major tuber proteins remained primarily in the polysome fraction with approximately 20% in the supernatant (data not shown). Evidence that the induced ADH and ALD RNAs were polysome bound is presented in Figures 7E and 7F: both species were displaced from the polysome

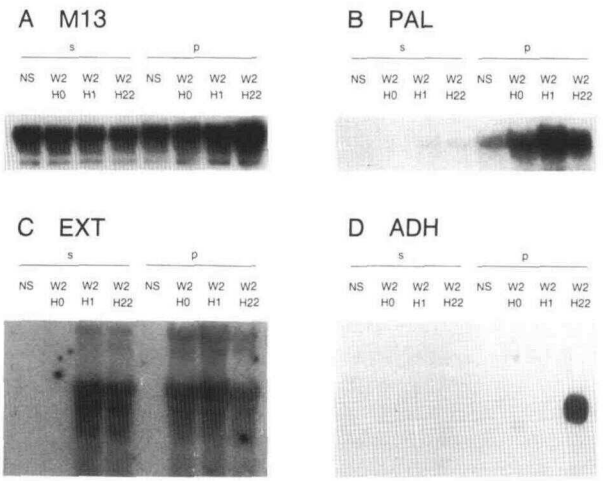

Figure 8. mRNA Associated with Polysomes Isolated from Wounded Hypoxic Tubers.

RNA extracted from the supernatant (s) and polysome (p) fractions of nonstressed tubers (NS), tubers wounded for 2 hr (W2 H0), or tubers subsequently incubated hypoxically for 1 hr (W2 H1) or 22 hr (W2 H22), was resolved by RNA gel blot hybridization to the ^{32}P-labeled probes indicated.

(A) Normalization control by hybridization to ^{32}P-M13 DNA.

(B) PAL probe.

(C) EXT probe.

(D) ADH probe.

fraction into the free RNA supernatant pool by incubation with 5 μg /mL puromycin. These results are in agreement with previous observations that indicated that mRNA of hypoxically induced genes are translated by 12 hr of onset of hypoxic stress (Vayda and Schaeffer, 1988).

Figure 8 shows that wound-response RNAs remained bound to polysomes throughout the period of hypoxic stress. It is known that wound-response RNAs are not induced when hypoxic tubers are subsequently wounded (Butler et al., 1990). However, RNAs induced by wounding before the onset of hypoxic conditions persist for up to 24 hr after transfer to hypoxic conditions, although their translation is inhibited within 30 min of hypoxic stress (Butler et al., 1990). As evident from Figure 8B, all of the PAL RNA present in wounded hypoxic tubers was found in the polysome fraction, even though the steady-state level of PAL RNA decreased between 12 hr and 24 hr of hypoxia (Figure 8B). Similarly, in Figure 8C, the majority of the EXT RNA present was in the polysome fraction. The proportion of EXT RNA present in the supernatant fraction was similar to that previously noted during aerobic, wounded conditions (Figures 1D and 1E). Thus, this observation was unlikely to be the result of a displacement of wound-response RNAs from polysomes when hypoxia-induced RNAs such as ADH (Figure 8D) became polysome associated. However, the wound-response RNAs were displaced from ribosomes by treatment with puromycin (data not shown). These data indicated that a mechanism must be operating in hypoxic tuber cells that arrests translation of polysomes to which wound-response RNA are bound.

Figure 9 shows the in vitro translation products generated by runoff of polysomes isolated from wounded tubers before and after hypoxic incubation. Polysomes isolated from tubers 4 hr after wounding were efficiently translated in vitro. Polysomes isolated from the same tuber 2 hr after transfer to hypoxic conditions (6 hr after the initial wounding event) generated the same products but at a much reduced level, approximately 5% of the aerobic sample. Polysomes isolated from tubers after longer hypoxic incubations (for example, 14 hr) were even less capable of producing runoff translation products in vitro. Essentially the same amount of PAL-hybridizing RNA was present in each of these samples (data not shown). Thus, polysomes

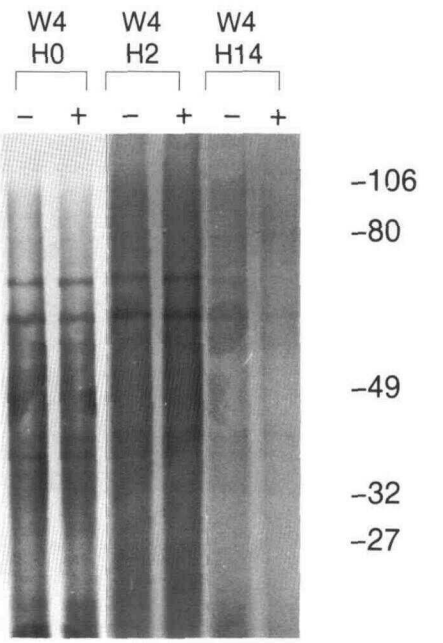

Figure 9. Translational Runoff in Vitro of Polysomes from Wounded Hypoxic Tubers.

Polysomes isolated from a tuber wounded for 4 hr, or the same tuber subsequently incubated hypoxically for 2 hr or 14 hr, were incubated with rabbit reticulocyte lysates in the absence (–) or presence (+) of the initiation inhibitor m^7 GMP. Ten micrograms of polysomal material was added to each reaction and incubated for 1 hr at 20°C. ^{35}S-methionine-labeled translation products were resolved by SDS-PAGE and fluorography. Sizes (in kilodaltons) indicated are relative to migration of known markers. Exposure time of the W4 H0 samples was one-fifth that of the other samples.

isolated from wounded hypoxic tubers exhibited the ability to produce translation products with SDS-PAGE mobilities similar to the products of wound-response polysomes but at significantly reduced levels. By contrast, synthesis of wound-response proteins was not detected in vivo at these times (data not shown; Vayda and Schaeffer, 1988; Butler et al., 1990).

The 32-kD Protein Remains Bound to Polysomes when Wounded Tubers Are Subsequently Incubated Hypoxically

Figure 10 shows that the 32-kD protein remained associated with the polysome fraction of wounded tubers when subsequently transferred to hypoxic conditions. The 32-kD protein was not apparent in SDS-PAGE gels of polysomal proteins from hypoxic tubers nor did it appear when hypoxic tubers were subsequently wounded (data not shown). Furthermore, the kinase activity that phosphorylated the 32-kD protein in response to wounding was also not detected in tubers incubated in hypoxic conditions (data not shown). Polysomes of wounded tubers transferred to hypoxic conditions exhibited minor polypeptides of 90 kD, 48 kD, and 45 kD, which may be characteristic of polysomes isolated from hypoxic tubers (Figure 10). However, it is clear that the 32-kD protein did not dissociate from polysomes upon shift to hypoxic conditions.

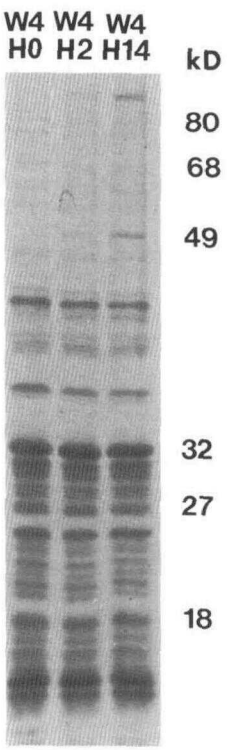

Figure 10. Polypeptides Associated with Polysomes Isolated from Wounded Hypoxic Tubers.

Polypeptides of the polysome fraction of a tuber wounded for 4 hr (W4 H0), and the same tuber subsequently incubated in hypoxic conditions for 2 hr (W4 H2) or 14 hr (W2 H14), were resolved by SDS-PAGE and staining with Coomassie blue. Sizes indicated are relative to migration of known markers.

DISCUSSION

We have identified two distinct mechanisms of translational regulation in potato tubers subjected to environmental stresses. Wounding effects dissociation and degradation of preexisting, tuberization-specific mRNAs from polysomes. By contrast, hypoxic stress inhibits translation of preexisting wound-response mRNAs without causing their dissociation from polysomes or degradation. Furthermore, a prominent 32-kD phosphoprotein has been noted that is specifically associated with polysomes isolated from wounded tubers.

The association of the 32-kD phosphoprotein with polysomes parallels the appearance and translation of wound-response mRNA species. It is possible that this protein mediates either the recognition of newly synthesized wound-response mRNAs or the inhibition of translation of the preexisting mRNAs that remain present in wounded tubers. Numerous potential mRNAs are present in wounded tubers that can specify production of polypeptides when deproteinized tuber RNA is translated in vitro. These species are not synthesized by wounded tubers in vivo (Vayda and Schaeffer, 1988; Butler et al., 1990). However, these species are also not efficiently translated by mature nonwounded tubers: polysomes isolated from nonwounded tubers allowed to complete translation in vitro generate runoff translation products comprising only the major proteins that accumulate in mature tubers and not these additional species. Thus, it is more likely that the 32-kD protein mediates recognition of wound-response mRNAs.

Other studies have detected modifications, such as phosphorylation, of ribosomal proteins during periods of selective translation (Fehling and Weidner, 1988). For example, Scharf and Nover (1982) described a 30-kD protein, identified as 40S ribosomal protein S6, that is rapidly dephosphorylated in tomato in response to heat shock, coincident with a global inhibition of protein synthesis. Similarly, Bailey-Serres and Freeling (1990) described a 31-kD phosphoprotein that is associated with polysomes during aerobic metabolism but is either not phosphorylated or not present when translation is inhibited by subjecting corn roots to hypoxic stress. However, the 32-kD protein we have identified in wounded potato tubers is not an

integral component of ribosomes because it can be dissociated by incubation with puromycin or Triton X-100. Conversely, this protein remains polysome associated when wounded tubers are transferred to hypoxic conditions. Thus, the ability to translate wound-response mRNAs and the failure to translate these mRNAs under hypoxic conditions cannot be explained solely on the basis of the presence or absence of the 32-kD protein. Thus, we conclude that the 32-kD protein is a marker for the presence of wound-response RNAs and may mediate their recognition by ribosomes but is not sufficient to specify their translation by ribosomes.

We cannot yet explain how translation of the wound-response mRNAs is inhibited upon the onset of hypoxic stress. The 90-kD, 48-kD, and 45-kD proteins detected in the polysome fraction of hypoxic tubers appeared after prolonged hypoxic incubation, 12 hr to 48 hr, and thus are unlikely to be involved in the inhibition of translation of wound-response mRNAs that occurs immediately upon onset of hypoxia. This inhibition is also unlikely to be the result of altered ATP levels. Although the ATP concentration of tuber tissue decreases by one-half within the first hour of hypoxic conditions, a similar drop in the ATP level occurs within 1 hr of wounding, a time at which translation in vivo is increased (J. Morelli and M. Vayda, unpublished results). Tubers incubated hypoxically for 24 hr exhibit an ATP level that is 10% that of aerobic tubers; however, at this time the hypoxically induced mRNAs are efficiently translated. Furthermore, individual amino acid pools do not fluctuate more than twofold and, thus, are unlikely to be limiting for protein synthesis (M. Vayda and H. Davies, unpublished observations). Finally, polysomes isolated from wounded tubers transferred to hypoxic conditions are inefficient at translation in vitro. The 5% to 20% sucrose gradient profile of polysomes from hypoxic, wounded tubers favors lower oligomers (data not shown); however, these complexes contain wound-response mRNAs in amounts comparable with polysomes isolated from aerobic wounded tissues. Thus, it is possible that unidentified factors may be preventing or arresting translation of wound-response mRNAs under hypoxic conditions. Conversely, this inhibition could result from the modification of dissociable factors that do not sediment into the polysome fraction and would not be detected in our analysis.

METHODS

Plant Material

Mature potato tubers (*Solanum tuberosum* cv Russet Burbank) were supplied by the Maine Agricultural Experiment Station and stored at 4°C. Before use, tubers were rinsed briefly with warm soapy water and surface sterilized with 5% hypochlorite (v/v). Tubers were dried and allowed to equilibrate to room temperature for 16 to 24 hr before experimentation.

Wounding and Hypoxic Conditions

Tubers were wounded using a P-200 micropipette tip, as described previously by Vayda and Schaeffer (1988). Hypoxic conditions were established by incubating tubers in a moist argon atmosphere, as described previously (Vayda and Schaeffer, 1988).

Phosphorylation of Proteins in Vivo

The 0.25 mCi of ^{32}P-orthophosphate (Du Pont-New England Nuclear) was provided to tuber wounds inflicted by a P-200 micropipette tip, either at the time of wounding or at the times afterward indicated in the figures. Label was incubated with tissue for 30 min. Approximately 3 g of tissue surrounding the wound site was removed with a 7-mm cork borer for polysome isolation. Labeling through eyebuds was accomplished by gently nicking the bud surface with a razor blade, providing 0.5 mCi of ^{32}P-orthophosphate to the depression surrounding each bud, and incubating for 1 hr. Label was quickly absorbed and transported vascularly to nearby tissue. Nonwounded or wounded tissue 0.5 to 1 cm from the labeling site was removed using the cork borer, with care taken to exclude the eyebud primary labeling site.

Polysome Isolation

Polysomes were isolated from tubers using a modification of the procedures described by Mignery et al. (1984) and Laroche and Hopkins (1987). Ground, frozen tissue was added to polysome buffer (200 mM Tris-HCl, pH 9.0, 400 mM KCl, 60 mM MgOAc, 50 mM EGTA, 250 mM sucrose, 0.01% Triton X-100, and 15 mM β-mercaptoethanol), clarified by centrifugation at 15,000*g*, and centrifuged through a 1.5 M sucrose cushion in a Beckman 70.1 Ti rotor at 45,000 rpm for 4.5 hr at 4°C. Forty nanograms of single-stranded M13 DNA was added to the supernatant and to the resuspended polysome pellet. The fractions were then extracted with phenol and precipitated with 0.5 M LiCl and 2.5 volumes of ethanol. Selected samples were incubated for 15 min with either 5 μg/mL puromycin at 37°C or 0.1% Triton X-100 at 4°C before centrifugation through the 1.5 M sucrose cushion.

Probes Used in RNA Gel Blot Analysis

The cDNA probe for PAL (Edwards et al., 1985) was kindly provided by Chris Lamb and Carole Cramer; the extensin probe (Chen and Varner, 1985) was the gift of Mary Tierney, Allan Showalter and Joseph Varner; the patatin probe (Mignery et al., 1984) was the generous gift of William Park, the PI-II probe (Graham et al., 1985) was kindly provided by Clarence Ryan; the aldolase cDNA (Kelley and Tolan, 1986) was the gift of Phil Kelley, and the ADH probe was generously provided by Judy Strommer.

Analysis of Polysomes

RNA recovered from the polysome and supernatant fractions was glyoxylated and resolved by electrophoresis through 1.5% agarose gels and by RNA gel blot hybridization (Maniatis et al., 1982). Samples were loaded such that each lane exhibited an M13 hybridization signal of equal intensity. Hybridization conditions for the probes used were as described by Butler et al. (1990).

Proteins associated with the polysome and supernatant fractions were resolved by electrophoresis through 15% SDS-PAGE gels (Vayda and Schaeffer, 1988). Equal volumes of materials were loaded per lane. ^{32}P-labeled proteins were detected by autoradiography of dried gels using Kodak X-AR5 film.

Translational runoff was performed as described by Berry et al. (1988; 1990). The polysome pellet from 3 g of tuber tissue was resuspended in 100 μL of translation cocktail (Du Pont-New England Nuclear) without ^{35}S-methionine. Ten micrograms of this material, typically 2 μL, was incubated with rabbit reticulocyte lysate, as described by the supplier (Du Pont-New England Nuclear), and incubated at either 20°C or 37°C for 60 min. Two hundred micromolar m^7 GMP and 25 μg/mL chloramphenicol were added to the (+) samples to prevent translation initiation and amyloplast translation, respectively. ^{35}S-methionine-labeled translation products were resolved by SDS-PAGE and fluorography using EN^{3}HANCE (Du Pont-New England Nuclear)..

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