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Reduction of Turgor Induces Rapid Changes in Leaf Translatable RNA[1]

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ABSTRACT

The turgor of pea (*Pisum sativum*) leaves was reduced by exposing excised pea shoots to a stream of 23°C air for 20 min. Poly(A)$^+$ RNA was isolated from control and wilted shoots, translated *in vitro* and radiolabeled translation products separated by electrophoresis on two-dimensional (isoelectric focusing-sodium dodecyl sulfate) polyacrylamide gels. This analysis showed that the levels of several poly(A)$^+$ RNAs increased in wilted plants. Most of the poly(A)$^+$ RNAs induced in wilted plants did not accumulate in response to heat shock or exogenously applied ABA even though endogenous ABA levels were found to increase in shoots 30 min after wilting and by 4 h had increased 50-fold (1 *versus* 0.02 microgram per gram fresh weight). A λgt10 cDNA library was constructed using poly(A)$^+$ RNA from wilted shoots which had been incubated for 4 hours. Differential screening of the library identified four clones corresponding to poly(A)$^+$ RNAs which are induced in wilted shoots.

Exposure of plants to water-limited environments can lead to leaf wilting, stomatal closure, and decreased photosynthesis (4, 24). Furthermore, leaf and reproductive tissue growth and development are often inhibited in water-deficient plants (3, 8). Some of these plant responses are the direct result of physical changes accompanying loss of cell turgor (6) while other responses could be due to altered levels of plant growth regulators. Plants exposed to water-limited conditions have been shown to have elevated levels of ABA (25) and ethylene (2) and reduced amounts of cytokinin (16) and gibberellin (1).

In *Escherichia coli*, changes in turgor pressure alter the expression of genes in the kdp operon which is involved in K^+ transport through the cell membranes (10). In addition, shifts in medium osmolarity modify the expression of OmpF and OmpC, *E. coli* genes which encode outer membrane porins (19). In plants, it is possible that changes in turgor are accompanied by rapid alterations of gene expression. Studies with inhibitors suggest that the increased ABA biosynthesis following plant turgor loss requires transcription of nuclear gene(s) (12) and cytoplasmic protein synthesis (12, 21). This implies that reduced cell turgor leads to changes in the population of translatable RNA. In this paper, we present evidence from two-dimensional gel electrophoresis of *in vitro* translated poly(A)$^+$ RNA and cDNA cloning experiments that several poly(A)$^+$ RNAs are rapidly induced when excised pea shoots are wilted.

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MATERIALS AND METHODS

Plant Growth and Treatments. *Pisum sativum* (Progress No. 9) was grown for 10 d at 23°C in well-watered vermiculite using a model E15 Conviron growth chamber with continuous illumination (70 $\mu E/m^2/s$) for 18 h followed by 6 h of darkness. Plant shoots (stems plus leaves) were cut, and the stem base was quickly submerged under water where a portion of the lower stem was excised. Each shoot was placed in a 0.5 ml microfuge tube containing 10 mM Hepes-KOH (pH 8.0) for 1 h pretreatment with illumination at 23°C. Shoots to be wilted were removed after pretreatment, weighed then wilted in darkness under a stream of air (23°C) for approximately 20 min until a 10 to 15% loss of fresh weight occurred. Wilted shoots were then incubated in a dark humid chamber for 0, 0.5, 1, 2, or 4 h prior to ABA assays and poly(A)$^+$ RNA isolation. For heat shock treatment, shoots were excised, placed in Hepes-pretreatment solution and treated for 1 h in the dark at 38°C. Stems were kept submerged to help prevent shoot dehydration and any wilted shoots were discarded. Shoots used for examining the effects of exogenous ABA were excised as above and placed in solutions of 10 mM Hepes-KOH (pH 8.0) which contained 18 μM (±) ABA. Shoots were treated for 1 or 4 h in the dark at 23°C.

ABA Assays. Plants were assayed for ABA content as previously reported (12). This involves extraction of 1 to 2 g fresh weight plant material in acetone:acetic acid (99:1; v/v) followed by sample cleanup by partitioning in ether at high and low pH and C_{18} reversed phase HPLC. ABA was quantified by Si column HPLC with a detection limit of approximately 4 pmol.

Poly(A)$^+$ RNA Isolation. Nucleic acids were isolated by phenol extraction and lithium chloride precipitation. Ten g of plant shoots were frozen in liquid N_2 and ground to a powder. Thirty ml of RNA extraction buffer (0.3 M NaCl, 1% SDS, 20 mM EDTA, 10 mM Tris-HCl pH 8.0) were mixed with the powdered tissue and stirred for 5 min. Thirty mL of ice-cold phenol and 30 mL of chloroform:isoamyl alcohol (23:1, v/v) were added to the mixture, allowing 5 min between additions (with occasional stirring). The mixture was centrifuged 5 min at 5000*g*, the aqueous phase reextracted with phenol and chloroform:isoamyl alcohol, and recentrifuged. The aqueous phase was adjusted to 4 M LiCl, 2 M urea, 1 mM EDTA and nucleic acids precipitated by adding 2.5 vol of ethanol and incubating overnight at −20°C. The nucleic acids were recovered by centrifugation, reextracted with phenol and chloroform:isoamyl alcohol and reprecipitated at −20°C with ammonium acetate and ethanol.

Poly(A)$^+$ RNA was isolated by oligo-dT cellulose (type 7, Pharmacia) affinity chromatography as described in Maniatis *et al.* (18) with one modification. During elution of the bound nucleic acid fraction, the elution buffer was preheated to 60°C and applied in 2 ml increments to the column. Following successive ethanol precipitations at −20°C, the poly(A)+ RNA was resuspended in water and stored at −80°C.

***In Vitro* Translations.** A nuclease-treated, amino acid depleted

wheat germ extract translation kit (Amersham Corp.) was utilized for *in vitro* translation. [^{35}S]Methionine (>800 Ci/mmol, New England Nuclear) was added to *in vitro* translations and translation conditions were optimized for K^+, poly(A)$^+$ RNA, and [^{35}S] methionine concentrations. The 30 μL reaction mixture contained 1 μg poly(A)$^+$ RNA, 50 μCi[^{35}S]methionine, 150 mM K^+, and 67 μM amino acids minus methionine and was incubated for 1 h at 25°C. A reaction typically yielded 80,000 to 150,000 cpm/μL of TCA precipitable material. Incorporation of reaction mixtures with no poly(A)$^+$ RNA added was 5,000 to 15,000 cpm/μl and resulted in only five detectable polypeptides following two-dimensional gel electrophoresis and autoradiography.

Gel Electrophoresis. Two-dimensional gel electrophoresis of *in vitro* translation products was performed according to O'Farrell (20) with modifications from Duncan and Hershey (9). Each gel contained 0.5 to 1.5 × 10^6 TCA precipitable cpm. Prior to electrophoresis, the 30 μL sample mix treated with 4 μg of DNAse and RNAse A for 15 min on ice, extracted with 1 vol phenol, and precipitated with 5 vol of 0.1 M ammonium acetate in methanol at −20°C overnight (14). The solution was centrifuged, the pellet washed three times with the ammonium acetate:methanol solution and once with acetone. The pellet was dried and resuspended in 30 μL of 9.5 M Schwartz-Mann ultrapure grade urea and 1 vol of lysis buffer (9.5 M urea, 2% v/v Nonidet P-40, 0.4% v/v LKB Ampholines [pH 3.5–10], 1.6% v/v LKB Ampholines [pH 5–7], 5% v/v β-mercaptoethanol). During the phenol extraction cleanup steps, approximately 10% of the total cpm could not be resuspended, but this treatment resulted in less gel background streaking and did not otherwise appear to alter the final profiles.

The second dimension SDS gels were run at a constant 2.5 W until the dye front moved within 1 cm of the gel bottom (usually 5–5.5 h). Gels were then fixed in aqueous methanol/acetic acid, fluorographed with 2,5-diphenyloxazole-DMSO, dried, and autoradiographed on Kodak XAR-5 film with a DuPont Cronix intensifying screen. Exposure times were normalized according to total cpm loaded per gel to give equivalent overall intensities on the autoradiograms but were generally for 6 d.

cDNA Cloning. Synthesis of double-stranded cDNA was done by the method outlined in the BRL cDNA Synthesis System Manual (Bethesda Research Laboratories) which was adapted from Gubler and Hoffman (11) to make use of cloned Moloney murine leukemia virus (M-MLV) reverse transcriptase. Internal *Eco*RI sites were methylated using *Eco*RI methylase (New England Biolabs), ends repaired with the Klenow fragment of *E. coli* DNA Polymerase I, and ligated onto [^{32}P]-labeled dephosphorylated *Eco*RI linkers (New England Biolabs). Following *Eco*RI digestion, phenol extraction, ethanol precipitation, and resuspension in 10 mM Tris-HCl, pH 7.8, and 1 mM EDTA, the cDNA was size fractionated on a 1 ml silated disposable pipet column using Bio-Rad Biogel A50M resin eluting with 150 mM ammonium acetate (15). Following agarose gel electrophoretic size analysis, fractions containing cDNA larger than 500 bp were selected for cloning into λgt10. These samples were lyophilized twice to remove ammonium acetate, pooled and resuspended in H_2O, and quantified by 5 min electrophoresis into a 3.5% polyacrylamide gel along with ΦX174 DNA fragments of known size and concentration and staining with Bio-Rad silver staining kit. The *Eco*RI digested dephosphorylated λgt10 cloning vector was ligated with the linkered-cDNA, the DNA packaged *in vitro* using Gigapack Gold and plated on C600 hfl—according to kit instructions (Vector Cloning Systems).

Library Screening. Nitrocellulose (Schleicher & Schuell) replica filters were prepared from the cDNA libraries as in Maniatis *et al.* (18). The adsorbed DNA was denatured by laying the filters (DNA side up) onto Whatman 3MM paper soaked in 1.5 M NaOH, 0.5 M Tris-HCl (pH 8.0), for 5 min, followed by 1.5 M NaCl, 0.5M Tris-HCl (pH 8.0), twice for 3 min, and 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) for 5 min. Filters were air dried 30 min then baked 2 h at 80°C.

cDNA probes were prepared from poly(A)$^+$ RNA isolated from control shoots, or shoots which were wilted and then incubated for 30 min or 4 h. Probes were synthesized with M-MLV reverse transcriptase using a 10 to 20 μL reaction containing 1 to 2 μg poly(A)$^+$ RNA, 100 μCi [α-^{32}P]dCTP (800 Ci/mmol), 200 units M-MLV reverse transcriptase and 0.5 μg oligo-dT_{12-18} (Pharmacia) per μg poly(A)$^+$ RNA, 0.5 mM dNTPs minus dCTP, and a reaction buffer of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.1 mg/ml BSA and 0.05 mg/ml actinomycin D. The mixture was labeled at 37°C for 1 h then chased 15 min after addition of 1 μL of 10 mM dNTPs. The reaction was stopped by adding 1.5 μL 0.5 M EDTA, 8 μL 0.2 M NaOH, and incubating 10 min at 70°C. Fifty μL of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA were added and the solution extracted once with TE-buffered phenol and chloroform:isoamyl alcohol (23:1, v/v) and once with chloroform:isoamyl alcohol. Unincorporated nucleotides were removed by spun column chromatography (18) and sample radioactivity determined by liquid scintillation counting in Aquasol (New England Nuclear).

Filters were prehybridized overnight at 45°C in 10 ml of 50% formamide, 9% polyethylene glycol-8000, 0.1 mg/ml boiled, sheared, salmon sperm DNA, 0.25 M NaCl, 1% SDS, 0.2% BSA, 0.2% PVP (mol wt 40,000), 0.2% Ficoll (mol wt 400,000), 50 mM Tris-HCl (pH 7.5), and 0.1% sodium pyrophosphate. Hybridizations were done overnight at 45°C using fresh solution as above with 5 to 20 × 10^6 cpm probe added. Filters were washed twice for 15 min at 23°C in 2× SSC, 0.5% w/v SDS, once for 30 min at 60°C in 0.1× SSC, 0.5% SDS, air dried, and autoradiographed. Clones of interest were transferred and eluted into 250 μL SM buffer (per L: 5.0 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 mL 1 M Tris-HCl (pH 7.5), 5 mL 2% gelatin), and 20 μL chloroform and rescreened after plating at a density of <200 plaques per plate.

DNA from clones which rescreened as wilting-induced positives was isolated using Lambdasorb (Promega) and subcloned into Bluescript SK plasmid (Vector Cloning Systems) following the supplier's procedures using DH5α as the host cell line. Recombinant plasmid DNA was prepared by an alkaline lysis mini-prep method similar to Maniatis *et al.* (18) except that the host cell pellet was digested with 20 mg/ml lysozyme prior to the alkaline-SDS incubation step. Two μg of *Eco*RI restricted plasmid were dot blotted onto GeneScreen Plus (New England Nuclear) according to the supplier's instructions. Replica filters were differentially screened as described above using cDNA probes synthesized from control shoot poly(A)$^+$ RNA or poly(A)$^+$ RNA from shoots which had been wilted and incubated for 0.5 or 4 h.

To determine how many unique cDNAs were present in the group of positives, the cDNA inserts were isolated from the recombinant plasmids by Tris-acetate agarose gel electrophoresis (18) and the Gene Clean kit (Bio 101) for DNA purification. The purified cDNA inserts were nick-translated using an Amersham nick-translation kit and [α-^{32}P]dCTP (800 Ci/mmol, New England Nuclear) and unincorporated nucleotides removed using spun column chromatography (18). Replicia filters containing individual dots of the entire group of 50 wilting-induced clones were made on GeneScreen Plus (New England Nuclear). Each nick-translated cDNA was tested for cross-hybridization to one replica filter using the conditions for prehybridization and hybridization described above.

Electrophoresis and Northern Analysis of RNA. Four unique clones were selected which gave a stronger hybridization signal to cDNA from shoots which had been wilted for 0.5 h and 4 h compared to cDNA from control shoots. These clones were

analyzed by Northern analysis as described in Maniatis *et al.* (18) utilizing formaldehyde gel electrophoresis of 1 μg control or 4 h wilted poly(A)$^+$ RNA. The RNA was transferred to GeneScreen membrane (New England Nuclear) using the membrane instruction manual's capillary blot procedure. Nick-translated cDNA insert from the selected wilting-induced clone was hybridized, the filters washed and autoradiography performed as described above.

Chemicals. Electrophoresis grade acrylamide and bis-acrylamide were obtained from Kodak. Unless noted otherwise, all enzymes were purchased from Bethesda Research Laboratories and all other chemicals from Sigma Chemical Co.

RESULTS

Wilting Treatment and Time Course of ABA Accumulation. It was previously shown that the turgor of pea leaves could be reduced from 0.6 MPa to 0.1 MPa within 20 min when excised pea shoots were exposed to a stream of 23°C air. When the wilted shoots were placed in a dark humid box leaf turgor remained constant for up to 4 h (12). The wilted leaves accumulated ABA and this accumulation was sensitive to pretreatment with inhibitors of transcription or cytoplasmic translation (12, 21). This result suggested that a change in poly(A)$^+$ RNA population may be required for the ABA increase accompanying plant wilting. Additionally, it was possible that other changes in gene expression occur in wilted plants. To test these possibilities, the time course of ABA induction and change in poly(A)$^+$ RNA due to leaf wilting was examined.

Pea shoots were excised and placed in 10 mM Hepes-KOH, pH 8.0 for 1 h to fully hydrate shoots. This pretreatment allowed us to determine if excision, in the absence of loss of water content, induced detectable changes in ABA (Fig. 1) or poly(A)$^+$ RNA (see below). Figure 1 shows that little ABA accumulated during the 1 h pretreatment. The pretreated shoots were then exposed to a stream of 23°C air until a fresh weight loss of 10 to 15% and wilting had occurred. To establish the ABA induction time course and maintain a constant low turgor condition, sets of treated plants were incubated in a humid chamber at 23°C in the dark for 0.5, 1, 2, and 4 h. Treatments were carried out in darkness to minimize water loss due to transpiration and synthesis of ABA due to light-induced carotenoid breakdown. Between 30 min and 1 h after reduction of shoot water content, ABA levels began to increase (Fig. 1). ABA levels continued to increase during a 4 h period reaching 1 μg ABA/g fresh weight of shoot tissue prior to dehydration.

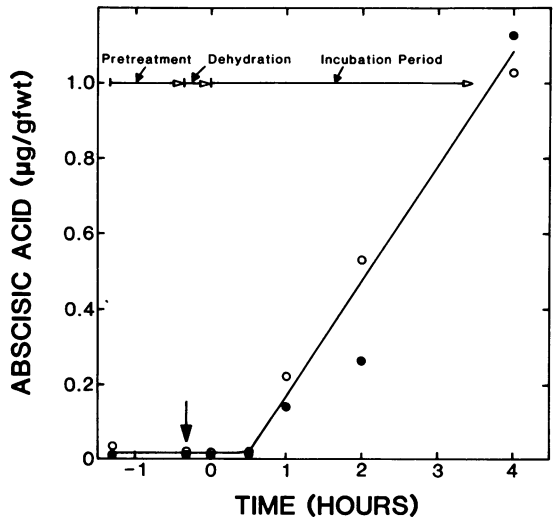


FIG. 1. Time course of changes in ABA content in excised pea shoots. All shoots were pretreated for 1 h in 10 mM Hepes-KOH (pH 8.0), dehydrated for 20 min and incubated for the indicated times. Solid symbols and open symbols represent two different experiments. The arrow in the bottom left marks the onset of drying treatment. Fresh weight was determined following the Hepes pretreatment.

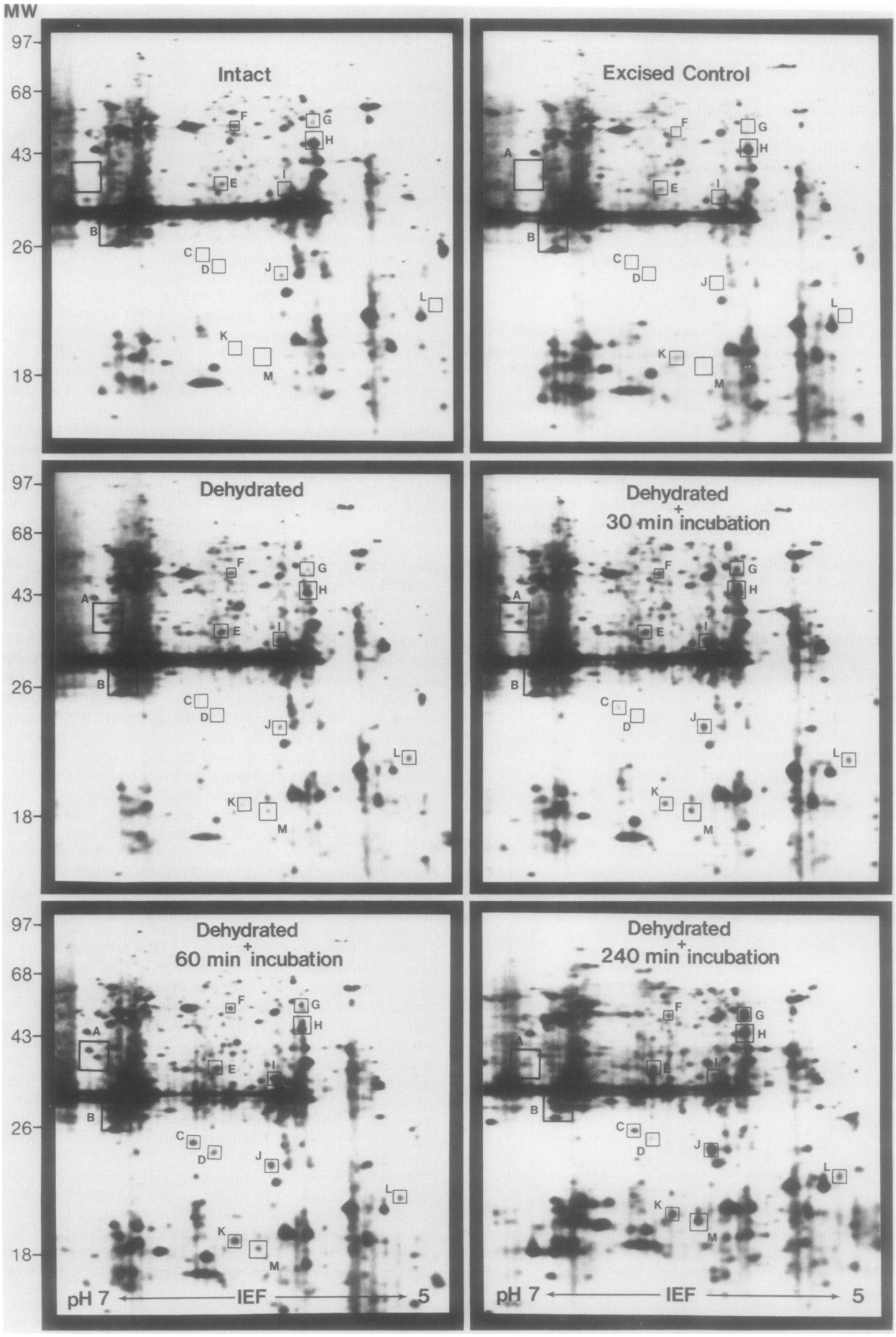
Changes in Poly(A)$^+$ RNA in Wilted Pea Shoots. Poly(A)$^+$ RNA was isolated from the shoots of pea plants grown in vermiculite and from excised pea shoots which had been treated as described above for the ABA induction study. The composition of poly(A)$^+$ RNA populations was analyzed by *in vitro* translation of poly(A)$^+$ RNA followed by separation of radiolabeled polypeptides on two-dimensional (IEF[2]-SDS) gels. The radiolabeled polypeptides were detected following fluorography and autoradiography (Figs. 2 and 3). This approach has been used in many previous studies to detect changes in poly(A)$^+$ RNA following perturbations such as exogenous auxin (23) or ABA (17) applications. In our studies, the analysis of RNA populations was done in duplicate. Two sets of plants were wilted and treated as described below and poly(A)$^+$ RNA isolated from each treatment. Each set of poly(A)$^+$ RNA was analyzed as described in the legend for Figure 2. Only minor quantitative differences were noted when comparing profiles from similar wilting treatments (for instance, when comparing autoradiograms of the 1 h treatment from the separate poly(A)$^+$ RNA isolations). Additionally, there were no qualitative differences noted during the comparisons between identical treatments.

Previous studies have shown that wounding or excision of plant organs can alter poly(A)$^+$ RNA populations (22). This raised the possibility that changes in RNA populations in excised, wilted shoots would reflect both the excision and wilting perturbations. To address this possibility, pea shoots of plants growing in vermiculite were excised, immediately frozen in liquid N_2, and poly(A)$^+$ RNA extracted. A second set of pea shoots was excised and the stems placed in pretreatment buffer for 1 h. After this treatment period, poly(A)$^+$ RNA was extracted from the pea shoots as described above. The two poly(A)$^+$ RNA populations are compared in Figure 2 ("Intact" and "Excised Control") where it is shown that excision caused by only minimal changes in the translatable RNA population detectable by this technique. Small changes in the levels of translation products labeled F, G, J, and K occurred during the pretreatment.

The next experiment was to determine if changes in poly(A)$^+$ RNA occurred when pretreated shoots were wilted and the time course of those changes. Therefore, poly(A)$^+$ RNA was extracted from a set of shoots immediately after the 20 min wilting period and also from plants which had been wilted and then incubated in a dark humid box for an additional 30, 60, and 240 min (Fig. 2). Analysis of the resulting poly(A)$^+$ RNA revealed that a subset of the RNA population increased in abundance in the wilted shoots compared to the excised control. The *in vitro* translation products which correspond to the induced RNAs are boxed in Figure 2 and are designated A to M. Several of the poly(A)$^+$ RNAs show increased abundance by the completion of the 20 min dehydration period (Fig. 2, areas A, E, F, I, J, L). Others are induced more slowly reaching their highest observed levels after 60 min (Fig. 2, areas C, D, H) or 240 min of incubation (Fig. 2, spots G, K, M). These results demonstrate that changes in translatable poly(A)$^+$ RNA occur in response to a decrease in shoot water content.

Some of the changes in poly(A)$^+$ RNA occur after ABA levels begin to increase in the wilted shoots and might be induced in response to increases in ABA. This would be consistent with previous reports of ABA-induced changes in poly(A)$^+$ RNA populations (13, 17). To test this possibility, shoots were excised and their stems placed in a 10 mM Hepes-KOH (pH 8.0) solution

[2] Abbreviation: IEF, isoelectric focusing.


MW
97
68
43
26
18
Intact
Excised Control
Dehydrated
Dehydrated
+
30 min incubation
Dehydrated
+
60 min incubation
Dehydrated
+
240 min incubation
A
B
C
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pH 7
IEF
5

FIGURE 2

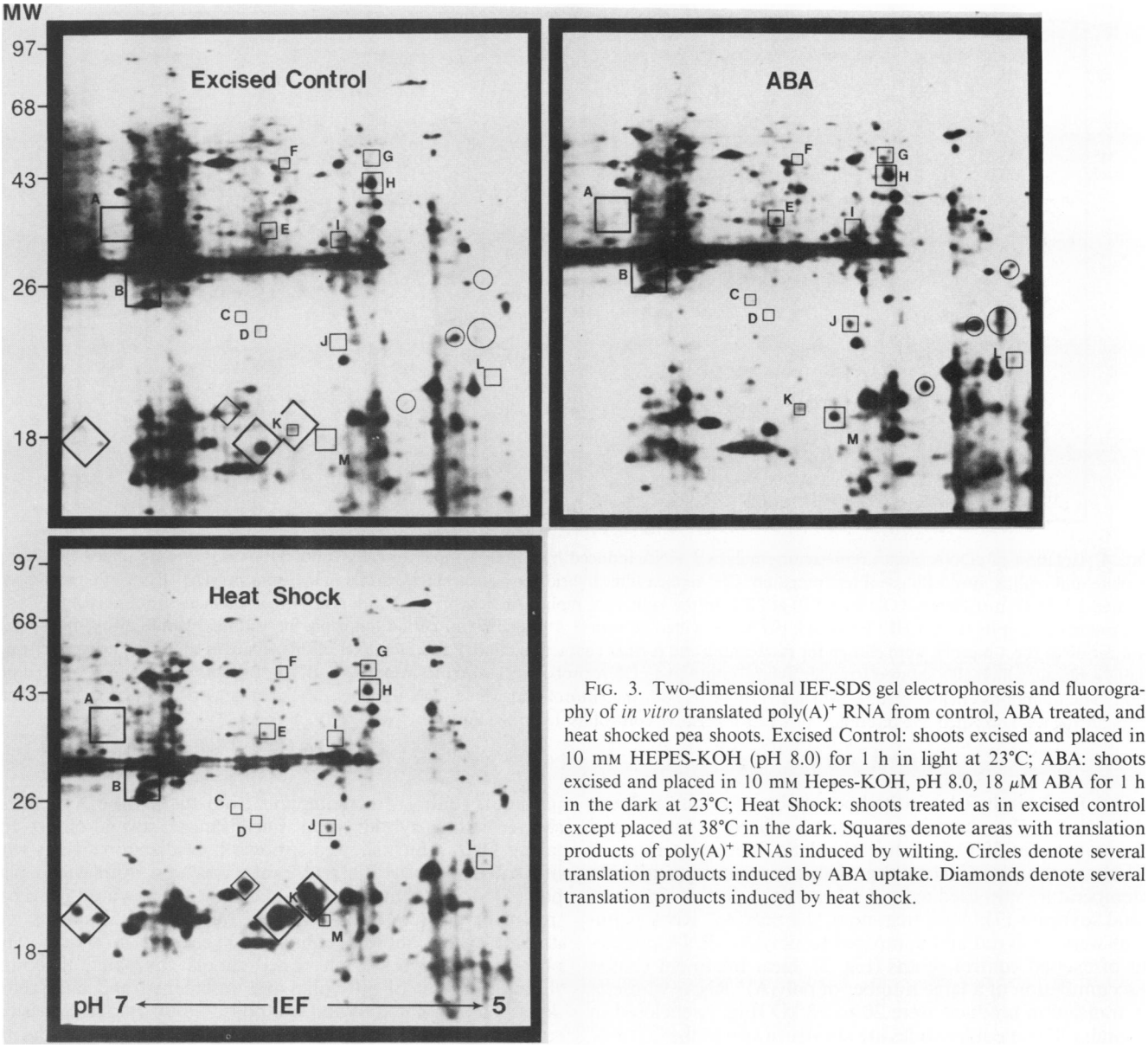

FIG. 3. Two-dimensional IEF-SDS gel electrophoresis and fluorography of *in vitro* translated poly(A)$^+$ RNA from control, ABA treated, and heat shocked pea shoots. Excised Control: shoots excised and placed in 10 mM HEPES-KOH (pH 8.0) for 1 h in light at 23°C; ABA: shoots excised and placed in 10 mM Hepes-KOH, pH 8.0, 18 μM ABA for 1 h in the dark at 23°C; Heat Shock: shoots treated as in excised control except placed at 38°C in the dark. Squares denote areas with translation products of poly(A)$^+$ RNAs induced by wilting. Circles denote several translation products induced by ABA uptake. Diamonds denote several translation products induced by heat shock.

containing 18 μM (±) ABA. Plants were allowed to take up ABA for 1 h or 4 h at 23°C in the dark. After incubation some of the shoots were extracted to determine ABA levels and other shoots were extracted for poly(A)$^+$ RNA. The ABA analyses showed that plants allowed to take up 18 μM (±) ABA either for 1 or 4 h had (±) ABA levels of 0.1 μg/g fresh weight in their shoots. Analysis of the poly(A)$^+$ RNA populations of these plants showed that a number of poly(A)$^+$ RNAs increased in abundance relative to their levels in excised control plants which had been incubated in 10 mM Hepes (pH 8.0) buffer only (Fig. 3). Two of the poly(A)$^+$ RNAs induced by this ABA treatment also were induced by wilting (Fig. 2 and 3, spots J and M). Other poly(A)$^+$ RNAs were induced by this ABA treatment but did not show large changes in wilted plants (Fig. 3, circles areas). Finally, most of the poly(A)$^+$ RNAs rapidly induced in wilted plants were not greatly affected during this ABA uptake experiment (Fig. 3, areas A, C, D, F, G, K, L). It is recognized that uptake of exogenous (±) ABA through the stem of a hydrated shoot may not induce all of the responses mediated by ABA in wilted shoots due to differences in compartmentalization and metabolism. However, the identification of poly(A)$^+$ RNAs which respond to exogenous ABA provides an indication that the corresponding genes may be similarly sensitive *in vivo* in wilted plants.

In an earlier study by Heikkila *et al.* (13), it was shown that a 70 kD heat shock protein mRNA was induced when maize plants were severely dessicated. Therefore, we tested whether the

FIG. 2. Two-dimensional IEF-SDS gel electrophoresis and fluorography of *in vitro* translated poly(A)$^+$ RNA from control and wilted pea shoots. RNA was translated using a wheat germ extract system and [^{35}S]methionine. Intact: shoots excised and quickly frozen in liquid N_2; Excised Control: shoots excised and placed in 10 mM Hepes-KOH, pH 8.0, for 1 h in light at 23°C; Dehydrated: shoots treated as in excised control then wilted by exposure to a 23°C air stream in darkness; Dehydrated + 30 min, 60 min, or 240 min incubation: treated as dehydrated shoots then 30, 60, or 240 min incubation in a dark humid chamber at 23°C. Squares denote areas with translation products of poly(A)$^+$ RNAs induced by wilting. Two sets of gels were generated from independent experiments with only minor quantitative differences noted. The gels represented here are from the same experimental set.

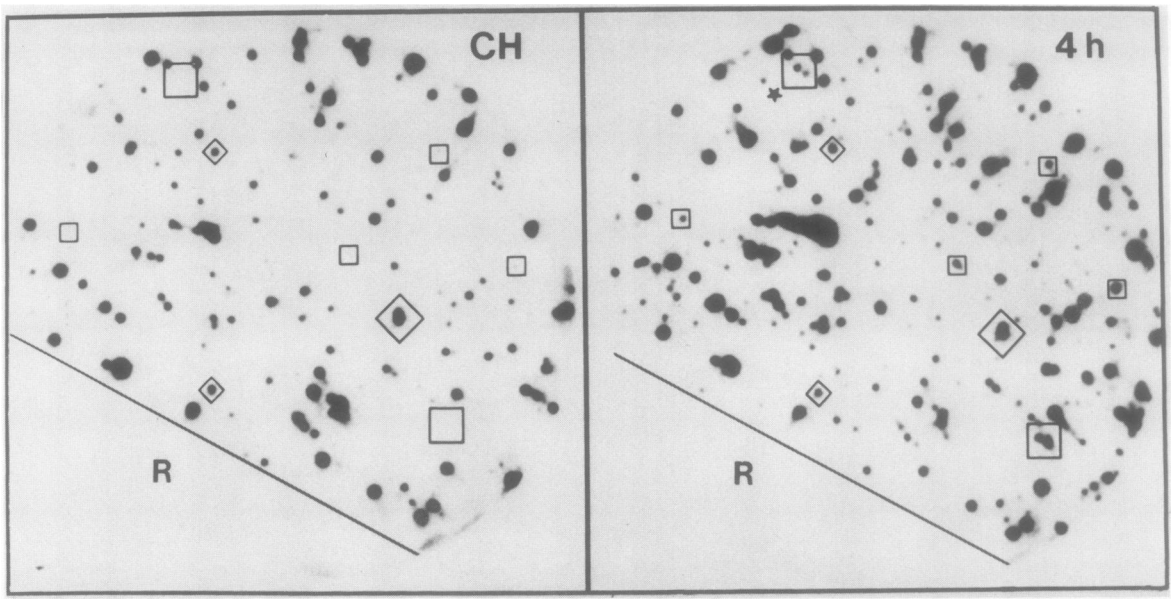

FIG. 4. Isolation of cDNA clones representing poly(A)$^+$ RNA induced by wilting. Approximately 2500 cDNA clones were plated per 85 mm petri plate and replica nitrocellulose filters prepared. CH: replica filter hybridized with a cDNA probe made from poly(A)$^+$ RNA of control shoots pretreated 1 h in 10 mM Hepes-KOH (pH 8.0) at 23°C in the light. 4 h: replica filter hybridized with a cDNA probe made from poly(A)$^+$ RNA of shoots pretreated 1 h in 10 mM HEPES-KOH, pH 8.0 + wilted 20 min + 4 h incubation period following the wilting. Plaques displaying stronger hybridization to the 4 h probe were chosen for rescreening and further characterization. Representatives of this set are in squares. The starred square contains a plaque which also showed stronger hybridization to a cDNA probe synthesized from poly(A)$^+$ RNA of shoots wilted and incubated only 30 min. The diamonds enclose plaques which hybridize to both the control and wilted cDNA probes. The area labeled R contains recombinant plaques having a 3 kbp Rheo test insert and provides a negative control for hybridizations.

poly(A)$^+$ RNAs induced in wilted pea shoots were also induced by heat shock. Pea shoots were excised, placed in buffer, and then incubated at 38°C for 1 h in the dark. This temperature is 15°C above the plant's normal growth temperature, similar to the temperature shift used to induce heat shock proteins in maize (5) and soybeans (7). After treatment, the poly(A)$^+$ RNA populations were analyzed and compared to poly(A)$^+$ RNA populations of excised control shoots (Fig. 3). Heat treatment caused the accumulation of a large number of poly(A)$^+$ RNAs whose *in vitro* translation products were 20 to 22 kD (Fig. 3, enclosed in diamonds). These polypeptides are similar in size to the 20 to 27 kD heat shock proteins studied by other investigators in maize (5) and soybeans (7). Although the major heat-induced poly(A)$^+$ RNAs did not correspond to RNAs induced by wilting, levels of a subset of the poly(A)$^+$ RNAs induced in wilted shoots (areas J and G) were elevated by heat treatment.

Detection of Poly(A)$^+$ RNAs Induced in Wilted Plants Using cDNA Libraries. Differential screening of a cDNA library was used as a second approach to determine if poly(A)$^+$ RNA levels for some genes changed in response to plant wilting. A cDNA library was prepared in λgt10 utilizing poly(A)$^+$ RNA isolated from pea shoots which had been wilted and then incubated for 4 h. From 5 μg of poly(A)$^+$ RNA, 1.5 μg of cDNA larger than 500 bp was synthesized. The library packaging yielded 4×10^7 plaques per μg cDNA and over 80% of the plaques contained cDNA inserts. Approximately 7000 clones of this library were replica plated and differentially screened with a cDNA probe synthesized from control shoot poly(A)$^+$ RNA or a cDNA probe synthesized from poly(A)$^+$ RNA of wilted shoots. An autoradiogram of one set of replica filters is shown in Figure 4. The lower portion of each filter (labeled R) contains plaques with λgt10 recombinants containing a 3 kbp Rheo test insert. As expected, the DNA in these plaques did not hybridize to the plant cDNA probe. In contrast, recombinants from the plant cDNA library showed strong hybridization. Furthermore, several clones contained DNA which showed stronger hybridization signals when incubated with a [^{32}P]cDNA probe prepared from wilted plant poly(A)$^+$ RNA compared to a cDNA probe prepared from control plant poly(A)$^+$ RNA (Fig. 4). Altogether, 50 clones were isolated which showed enhanced hybridization signals when probed with cDNA from wilted shoots compared to control shoots. This set of 50 clones was rescreened and analyzed as described in "Materials and Methods." Four unique clones corresponding to inducible poly(A)$^+$ RNAs (clones C–F) and two constitutive clones (clones A, B) were selected. Northern analyses were done using equal amounts of poly(A)$^+$ RNA isolated from control shoots and shoots which had been wilted and incubated for 4 h (Fig. 5). The results in Fig. 5 show that poly(A)$^+$ RNA which hybridizes to clones C, D, E, and F is induced several-fold in wilted shoots compared to hydrated shoots.

DISCUSSION

Several studies have shown that auxin (23) or heat shock (7) can cause rapid changes in plant gene expression. These changes were detected using *in vitro* translation and IEF-SDS gel analysis of translatable RNA and differential screening of cDNA libraries. In the present study we employed similar techniques to determine if changes in poly(A)$^+$ RNA abundance occur in response to decreased cell turgor. Our results from the two-dimensional gel electrophoresis analysis show that the levels of at least 13 poly(A)$^+$ RNAs are induced when pea shoots are wilted. This number is a minimum estimate and likely represents changes in abundant poly(A)$^+$ RNAs due to the detection limit of these techniques.

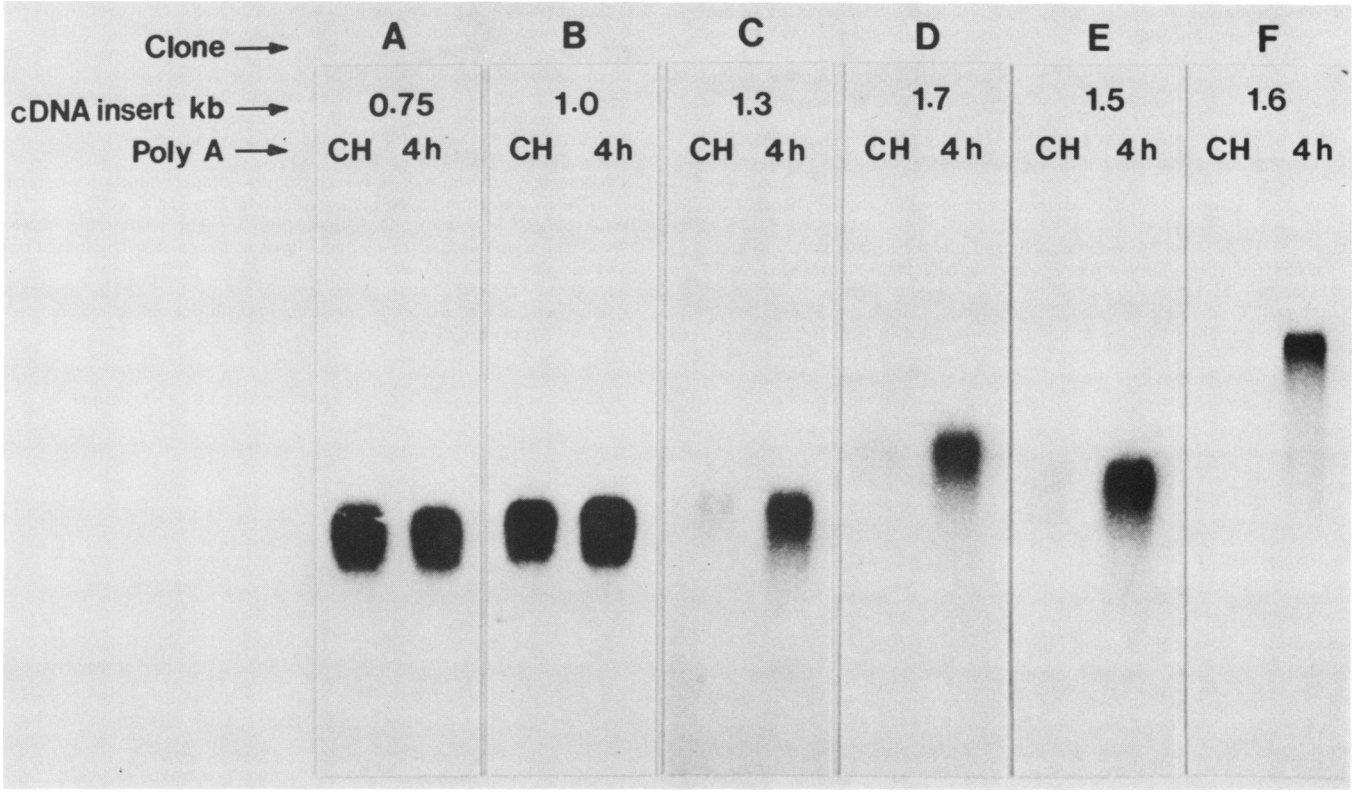

FIG. 5. Northern analysis of wilting-induced clones using control and wilted shoot poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated from control or wilted shoots, analyzed by 0.8% agarose formaldehyde gel electrophoresis and transferred to GeneScreen membrane. cDNA inserts from selected wilting-induced clones were nick-translated and hybridized to the transferred RNA, and the filters washed and autoradiographed as described. CH: poly(A)$^+$ RNA from shoots excised and placed in 10 mM HEPES-KOH, pH 8.0, for 1 h in light at 23°C; 4 h: poly(A)$^+$ RNA from shoots treated as in CH then wilted by exposure to a 23°C air stream for 20 min in darkness and incubated for 4 h in a dark humid chamber at 23°C. Clones C to F were selected as 4 h wilting-induced by the differential screening process described in the text. Clones A and B were selected as noninduced control clones by the same process. Filters for clones C to F contain 1 μg poly(A)$^+$ RNA per lane. Filters for clones A and B contain 0.6 μg poly(A)$^+$ RNA per lane.

The observed changes in translatable RNA were induced by wilting and not by shoot excision or, with two exceptions, by elevated temperature. Furthermore, the abundance of several of the poly(A)$^+$ RNAs increased by the end of the 20 min dehydration treatment. Other poly(A)$^+$ RNAs began to increase 30 min after dehydration, about the time ABA levels started to increase in the wilted shoots. A subset of these changes could be induced by addition of exogenous ABA to well-watered shoots (Fig. 3, spots J and M). Interestingly, the level of several poly(A)$^+$ RNAs that were increased in well-watered shoots treated with exogenous ABA were not induced in wilted plants which had similar levels of ABA. This can be seen by comparing the ABA gel profile of Fig. 3 to the 30 min and 60 min incubation profiles of Figure 2. The time course study (Fig. 1) showed that between 30 and 60 min following wilting, the ABA concentration in the shoots reached 0.1 μg/g fresh weight, similar to the overall level in the shoots which were incubated in the 18 μM (±) ABA solution for 1 h in the dark. Of course, the kinetics associated with the uptake of 18 μM (±) ABA solution and the distribution of this ABA is probably different from the kinetics of induction and distribution of endogenous ABA synthesized in the wilted shoots.

It seems likely that some of the changes in poly(A)$^+$ RNA observed in this study may be involved in responses to wilting such as growth inhibition, osmoregulation or ABA biosynthesis. Further studies on the turgor responsive genes identified in this study are in progress to establish the function of the gene product and mechanism(s) which regulate these genes.

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