PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Auxin-Regulated Gene Expression

J. L. Key, P. Kroner, J. Walker, J. C. Hong, T. H. Ulrich, W. M. Ainley, J. S. Gantt and R. T. Nagao

Phil. Trans. R. Soc. Lond. B 1986 **314**, 427-440
doi: 10.1098/rstb.1986.0063

Phil. Trans. R. Soc. Lond. B **314**, 427–440 (1986)
Printed in Great Britain

Auxin-regulated gene expression

By J. L. Key, P. Kroner, J. Walker, J. C. Hong, T. H. Ulrich, W. M. Ainley, J. S. Gantt and R. T. Nagao

Botany Department, University of Georgia, Athens, Georgia 30602, *U.S.A.*

During the 1960s a wide range of studies provided an information base that led to the suggestion that auxin-regulated cell processes – especially cell elongation – may be mediated by auxin-regulated gene expression. Indirect evidence from our work, based on the influence of inhibitors of RNA synthesis (e.g. actinomycin D) and of protein synthesis (e.g. cycloheximide) on auxin-induced cell elongation, coupled with correlations of the influence of auxin on RNA synthesis and cell elongation, provided the basis for this suggestion. With the availability of techniques for DNA–DNA and DNA–RNA hybridization, mRNA isolation–translation, *in vitro* 2D gel analysis of the translation products, and ultimately the cloning by recombinant DNA technologies of genomic DNA and copy DNAs (cDNAs) made to poly(A)$^+$ mRNAs, we and others have provided direct evidence for the influence of auxin on the expression of a few genes (i.e. poly(A)$^+$ RNA levels). Our laboratory has provided evidence for auxin's both down-regulating and up-regulating the level of a few poly(A)$^+$ mRNAs out of a population of about 4×10^4 sequences that are not significantly affected by auxin. In our studies on auxin-regulated cell elongation, two cDNA clones (pJCW1 and pJCW2) were isolated which corresponded to poly(A)$^+$ mRNAs that responded during growth transitions in a way consistent with a potential role of their protein products in cell elongation. These mRNAs are most abundant in the elongating zone of the soybean hypocotyl. Upon excision and incubation in the absence of auxin, these mRNAs deplete in concert with a decreasing rate of cell elongation. Addition of auxin to the medium results in both increased levels of these mRNAs and enhanced rates of cell elongation. These mRNAs do not deplete if auxin is added to the medium at the onset of excised incubation, and cell elongation rates remain high. We have isolated and sequenced genomic clones that are homologous to these cDNAs. Of the two genes sequenced, both genes are members of small multigene families. There are regions of high amino acid homology even though the nucleotide sequences are sufficiently different in these regions for cross-hybridization of the clones not to be observed. More recently others, especially Guilfoyle's laboratory, have shown that auxin selectively and rapidly influences the level of certain mRNAs and proteins. We have worked on other gene systems such as ribosomal proteins and possible cell wall proteins that are responsive to auxin; again the nature of regulation of expression of these genes is not known. The question now is not whether auxin selectively alters gene expression and thus the biology of responsive tissues, but what the mechanism is by which auxin alters expression of specific genes. Additionally, the function of these gene products remains a 'mystery'. Fortunately, experimental approaches are available to answer these questions and are actively being pursued.

Introduction

The study of auxin-mediated changes in RNA metabolism dates back to the early work from Skoog's laboratory (see Skoog 1954). Subsequently, several laboratories demonstrated marked changes in the RNA content of a wide range of plant tissues following auxin treatment (see Trewavas 1968; Key 1969; Hanson & Slife 1969). Several lines of evidence were suggestive

of a close relationship between auxin-mediated changes in growth patterns and altered RNA metabolism:

(1) In mature regions of the soybean hypocotyl, auxin induces a rapid and large accumulation of RNA (Key & Shannon 1964; Key *et al.* 1966).

(2) Auxin enhances the incorporation of radioactive nucleotides into RNA of both excised elongating and mature hypocotyl in a concentration-dependent manner. Although rRNA reflects the major accumulating class of RNA, the synthesis of poly(A)$^+$ RNA (and other AMP-rich RNAs) is significantly enhanced (see Key & Ingle 1968).

(3) Inhibitors of RNA synthesis (e.g. actinomycin D) and of protein synthesis (e.g. cycloheximide) are effective in suppressing auxin-induced cell elongation (see, for example, Key 1964). Inhibition of an increment of poly(A)$^+$ RNA and/or protein synthesis by these agents leads to a comparable incremental inhibition of auxin-induced cell elongation (Key *et al.* 1967).

(4) With the simultaneous addition of auxin and actinomycin D, for example, endogenous cell elongation is not significantly impaired for 1–2 h whereas auxin-induced growth is markedly inhibited. Thus auxin cannot 'amplify' the endogenous growth potential of this tissue in the absence of RNA and protein synthesis (see, for example, Key *et al.* 1967). However, if auxin is added to establish the maximum growth rate before the addition of actinomycin D, auxin-induced growth is inhibited with similar kinetics as the endogenous growth (i.e. absence of added auxin).

These observations led to the concept of 'growth-limiting' RNA(s) and protein(s) and were suggestive, coupled with the above noted results, of an auxin-induced increase in these 'growth-limiting' macromolecules (see, for example, Key *et al.* 1967; Key 1969; Vanderhoef 1980).

Technologies were not available in the late 1960s and early 1970s to permit an analysis of the relation of auxin-induced growth processes to auxin-mediated changes in RNA and protein metabolism beyond these indirect and correlative observations. Further, theoretical considerations of Evans & Ray (1969) led those investigators to conclude that auxin could not possibly mediate enhanced rates of cell elongation via mechanisms involving auxin-enhanced rates of synthesis of specific mRNAs. Some of their assumptions (e.g. times required by plant cells to elaborate new RNA molecules and the presumed half lives of those molecules, kinetics of auxin-enhanced RNA synthesis, and possibly even kinetics of auxin-induced sustained growth excluding the rapid, short-lived burst of growth that is not auxin-specific; see Vanderhoef (1980) for elaboration of this latter point) have proved to be too restrictive or incorrect. None the less, these considerations contributed to a significantly reduced scientific effort designed to unravel the question of the relation of auxin-enhanced rates of cell elongation to auxin-mediated changes in RNA–protein synthesis. Additionally, the merging excitement over auxin-induced proton extrusion and the acid growth hypothesis (see discussion by Vanderhoef (1980) and Evans (1985)) attracted the attention of many plant biologists interested in auxin 'action'. This hypothesis of auxin action has been brought into question by a number of studies, and especially by the results published by Terry & Jones (1981) which demonstrated the dependence of external K^+ and Ca^{2+} ions for auxin-induced proton extrusion while auxin-induced rates of cell elongation were similar in the absence or presence of these ions. The two-component aspect of the auxin-induced enhancement of cell elongation (see Vanderhoef 1980) also raised considerable doubt about interpretation of kinetic relations of the growth response to auxin

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

as related both to 'acid growth' and 'RNA synthesis-dependent growth'. Other considerations of auxin, cytokinin and fusicoccin effects on cell elongation also raise questions about this mechanism of auxin-regulated cell elongation.

Auxin-induced changes in poly(A)$^+$ RNA levels in mature soybean hypocotyl

Technological advances in the 1970s, especially recombinant DNA approaches, methods for RNA–DNA hybridization analyses, and *in vitro* translation of poly(A)$^+$ RNAs followed by 2D gel analysis of the products, led to possible new approaches to assess auxin-induced changes in specific mRNAs as opposed to general effects as assessed in the early investigations on RNA synthesis. Thus a number of studies based on these new technologies have permitted the demonstration of rapid and specific increases and decreases in some mRNAs after auxin treatment (see, for example, Baulcombe *et al.* 1980, 1981; Baulcombe & Key 1980; Walker & Key 1982; Walker *et al.* 1985; Zurfluh & Guilfoyle 1982*a*, *b*; Hagen *et al.* 1984; Hagen & Guilfoyle 1985; Theologis *et al.* 1985). A brief description of some of these results follows. Guilfoyle (1986) has recently reviewed and analysed the literature related to auxin-regulated gene expression.

The complexity of total poly(A)$^+$ RNA is not significantly affected by auxin treatment of mature soybean hypocotyl tissue (Baulcombe *et al.* 1980, 1981) as assessed by kinetic hybridization analyses of copy DNA (cDNA) to poly(A)$^+$ RNA in excess or by saturation hybridization of single copy DNA to poly(A)$^+$ RNA in excess. The kinetic hybridization analyses demonstrated changes in relative concentration of some sequences including an especially abundant component of poly(A)$^+$ RNA in control tissue that decreases markedly after auxin treatment. *In vitro* translation in a wheat-germ system of poly(A)$^+$ RNAs isolated from mature tissue followed by resolution of the translation products on O'Farrell 2D gels indicate that about 40 abundant poly(A)$^+$ RNAs changed in concentration after auxin treatment, with about equal numbers showing an increase and a decrease. Zurfluh & Guilfoyle (1982*b*) have found that auxin alters the level of translatable mRNAs similarly in excised and intact mature soybean hypocotyls.

Based on the observation from hybridization analyses, that auxin treatment caused a depletion of a very abundant component of poly(A)$^+$ RNA in mature soybean hypocotyl, cDNAs made to control poly(A)$^+$ RNA were cloned and recombinant colonies screened with radiolabelled cDNAs made to poly(A)$^+$ RNAs from control and auxin-treated tissue (Baulcombe & Key 1980). Several cDNA clones representative of three different poly(A)$^+$ RNAs that responded rapidly to auxin treatment were isolated. These clones represented three very abundant poly(A)$^+$ RNA sequences of mature control tissue (about 2.1% of the total). The concentration of each of these sequences decreased markedly and rapidly, representing only about 0.01–0.05% each of the total poly(A)$^+$ RNA after 24 h of auxin treatment. Most of this decrease occurs over the initial 8–12 h after auxin treatment, before the onset of auxin-induced cell division (figure 1). These studies provided the first direct demonstration that auxin modulates the levels of specific mRNAs. The mechanism by which auxin regulates the concentration of these poly(A)$^+$ RNAs is not known, but both transcriptional and post-transcriptional events may be involved. Additionally, the data of figure 1 demonstrate an

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

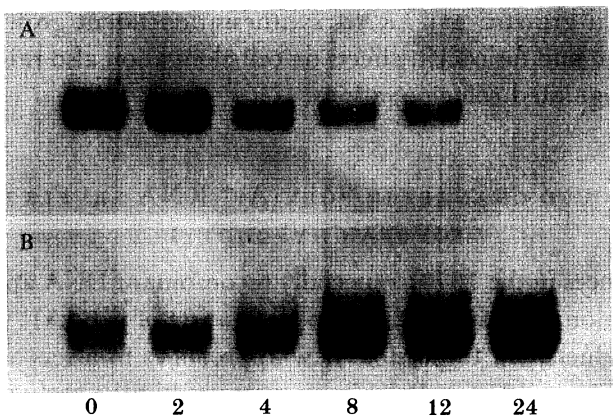


FIGURE 1. Effect of auxin on the concentration of two soybean mRNAs. Northern analysis of poly(A)$^+$ RNA isolated from auxin-treated 4-day-old etiolated mature hypocotyl. The blots were probed with cDNA inserts labelled by nick-translation. A, Probed with cDNA p11 (Baulcombe & Key 1980); B, probed with cDNA encoding cytoplasmic ribosomal protein L13 (Gantt & Key 1985); numbers indicate time (hours) of auxin treatment, respectively. (Data of P. Kroner & J. L. Key.)

inverse relation after auxin treatment between the decrease in concentration of these three abundant sequences described by Baulcombe & Key (1980) and the enhanced accumulation of ribosomal protein mRNAs (see Gantt & Key 1985). The accumulation of ribosomal protein mRNAs in response to auxin closely parallels the auxin-enhanced transcription of rRNA genes and subsequent ribosome accumulation (Guilfoyle *et al.* 1975; Gantt & Key 1985).

The three abundant poly(A)$^+$ RNAs of mature hypocotyl that are down-regulated by auxin treatment each have unique patterns of developmental expression (P. Kroner & J. L. Key, unpublished data). The relative steady-state level in various soybean tissues of the mRNA homologous to one of these clones, designated p6, is presented in figure 2. There are significant differences in the steady-state level of this mRNA in different tissues of soybean. The most dramatic difference correlates with the more mature tissues of seedlings with high relative concentrations of the sequence while highly meristematic tissues (zones of active cell division), such as the root tip, auxin-treated mature hypocotyl, and log phase cells in culture, have barely detectable levels of the sequence. Although there are some differences, this general pattern is observed for each of the three classes of sequences homologous to the cDNA clones isolated by Baulcombe & Key (1980).

Hagen *et al.* (1984) isolated four different cDNA clones from soybean hypocotyl poly(A)$^+$ RNA after 2 h of auxin treatment that are up-regulated. These poly(A)$^+$ RNAs increase in concentration rapidly after auxin treatment from very low or undetectable levels to maximum levels within about 2 h (see figure 3). The concentration of these mRNAs decreases dramatically after 24 h of auxin treatment of the intact seedling. These poly(A)$^+$ RNAs respond similarly to a number of different active auxins, but not to the ethylene precursor, ethephon, or to inactive auxin analogues. RNAs homologous to these cDNAs also increase in concentration after auxin treatment of both mature and elongating excised hypocotyl tissue. These two sets of data (Baulcombe & Key 1980; Hagen *et al.* 1984) directly demonstrate by cDNA–Northern and dot hybridization both up-regulation and down-regulation of poly(A)$^+$ RNA levels by auxin and are consistent with observations based on 2D gel analyses of translation products of poly(A)$^+$ RNA from mature soybean hypocotyl (see, for example, Baulcombe *et al.* 1980; Zurfluh & Guilfoyle 1982*b*).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

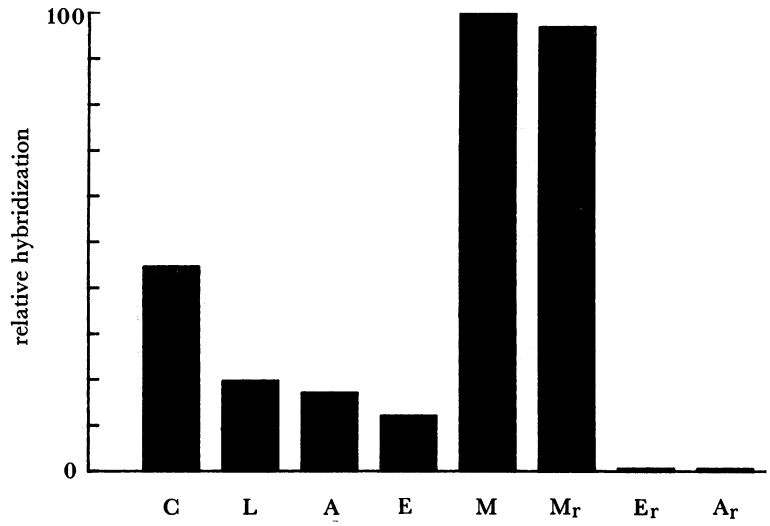


FIGURE 2. Relative hybridization of cDNA p6 to poly(A)$^+$ RNA isolated from various soybean tissues. Tissue designated as C (cotyledons), L (primary leaves), A (apical meristem), E (elongating hypocotyl) and M (mature hypocotyl) was excised from 4-day-old etiolated seedlings. Root tissue (M_r, mature root; E_r, elongating root; A_r, apical root meristem) was excised from seedlings grown in bags for 2 days. Values were determined by dot-blot analysis with the use of equivalent amounts of poly(A)$^+$ RNA isolated from each tissue hybridized to nick-translated cDNA. The dots were counted in a liquid scintillation counter and each count min^{-1} μg^{-1} poly(A)$^+$ RNA was normalized for the bar graph as a percentage of the mature hypocotyl tissue. (Data of P. Kroner & J. L. Key.)

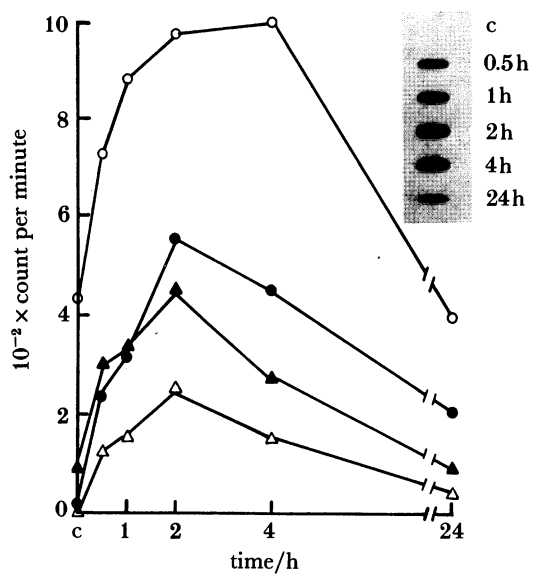


FIGURE 3. Hybridization analysis of cDNA plasmids to RNA from untreated (c) and 2,4-D-treated intact soybean hypocotyl. After treatment of the hypocotyl with 2,4-D (for 0.5, 1, 2, 4 or 24 h), poly(A)$^+$ RNA was extracted; 2 μg of RNA was denatured, 'slot-blotted' onto nitrocellulose and hybridized to labelled cDNA plasmids. The autoradiogram of the hybridization of pGH3 to the slot-blot is shown (inset). Radioactivity associated with individual slots was determined by scintillation counting and the activity (the average of duplicate slots) plotted (○, pGH1; △, pGH2; ●, pGH3; ▲, pGH4). (From Hagen *et al.* (1984).)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Hagen & Guilfoyle (1985) have shown that the increase in concentration of the poly(A)$^+$ RNAs homologous to the four cDNA clones isolated by Hagen *et al.* (1984) results from an enhanced rate of transcription of the genes after auxin treatment (figure 4). The rates of transcription of the auxin-responsive sequences were 10- to 100-fold greater with nuclei isolated from auxin-treated plumules than with those from untreated control plumules. Increased rates of transcription were also observed when nuclei were isolated from hypocotyls of auxin-treated soybean seedlings and auxin-treated excised hypocotyl sections. The enhancement of transcription of these genes was very rapid and was not dependent upon protein synthesis. Total RNA synthesis (precursor incorporation) by these nuclei was not significantly affected by auxin over the time periods analysed. Enhanced rates of transcription of two of the poly(A)$^+$ RNAs showed very high specificity for an active auxin, whereas transcription of the other two showed less specificity for an active auxin.

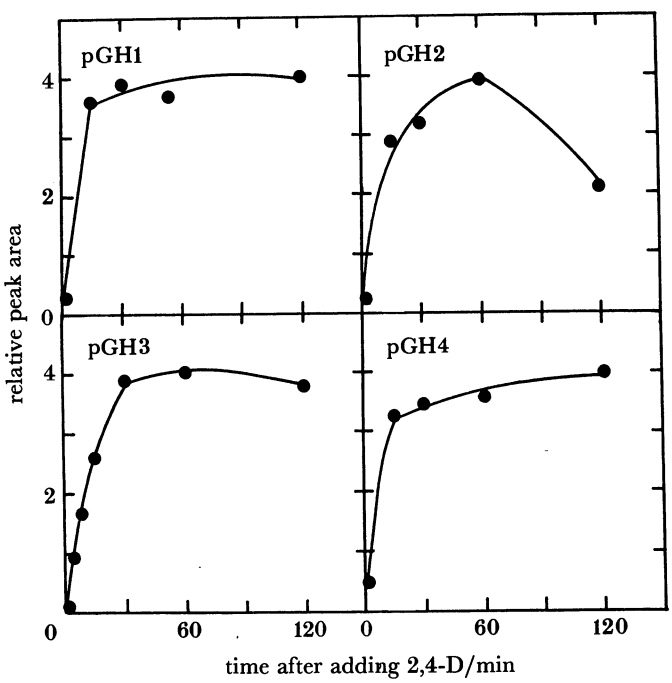


FIGURE 4. Induction kinetics of 2,4-D-regulated transcription. Strand-specific M13 clones of pGH1, pGH2, pGH3, and pGH4 were slot-blotted and hybridized to ^{32}P-labelled nuclear transcripts from soybean plumules incubated with 10^{-4} M 2,4-D for the times indicated. Hybridization was quantitated by densitometry. (From Hagen & Guilfoyle (1985).)

CHANGES IN POLY(A)$^+$ RNAS DURING AUXIN-INDUCED CELL ELONGATION

Because auxin is classically defined as a hormone that controls cell elongation, a series of experiments were designed to isolate cDNA clones to poly(A)$^+$ RNAs that increased in concentration after auxin treatment of excised elongating hypocotyl tissue (Walker & Key 1982). The rationale was to clone cDNA made to poly(A)$^+$ RNA of the elongating region of the soybean hypocotyl without any incubation, because this tissue presumably contains all mRNAs necessary for maximum rates of elongation. The library was then screened with cDNAs made to (1) poly(A)$^+$ RNA isolated from excised tissues in which the rate of elongation had decreased dramatically compared with intact tissue (i.e. 4 h of incubation in the absence of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

added auxin), and (2) poly(A)$^+$ RNA isolated from excised tissues incubated as above but in the presence of added auxin, which enhanced the elongation to rates similar to that of intact tissue. Two cDNA clones, designated pJCW1 and pJCW2, were isolated (Walker & Key 1982) that responded in a manner expected of mRNAs involved in the rate-limiting process(es) of cell elongation.

Early results suggested that the rate-limiting RNAs and encoded proteins for cell elongation had half-lives of about 1–2 h (see, for example, Key & Ingle 1964) or were even 'used up' as with possible structural proteins. The levels of the poly(A)$^+$ RNAs homologous to pJCW1 (JCW1 RNA) and pJCW2 (JCW2 RNA) are higher in the elongating tissue than in dividing or mature tissues, and JCW1 RNA is present at higher levels than JCW2 RNA (Walker & Key 1982). Both sequences deplete rapidly during incubation of excised sections in the absence of exogenous supplies of auxin; JCW1 RNA is maintained at a near-constant level when auxin is present throughout the incubation of the elongating sections, whereas the amount of JCW2 RNA increases above the level in intact tissue. The addition of auxin to excised tissues that were depleted in JCW1 and JCW2 sequences by a 4–6 h incubation without an exogenous auxin supply resulted in a rapid accumulation of both poly(A)$^+$ RNAs. The JCW1 sequence increased significantly within 30 min and continued to increase up to 90 min of incubation to levels threefold to fivefold above the auxin-depleted level and to about the levels in intact tissue. The JCW2 RNA responded very rapidly to auxin treatment, with increases being detectable within 15 min (the shortest time measured); JCW2 RNA increased fivefold to tenfold within 30–60 min to levels even higher than in intact elongating hypocotyl tissue (see figure 5).

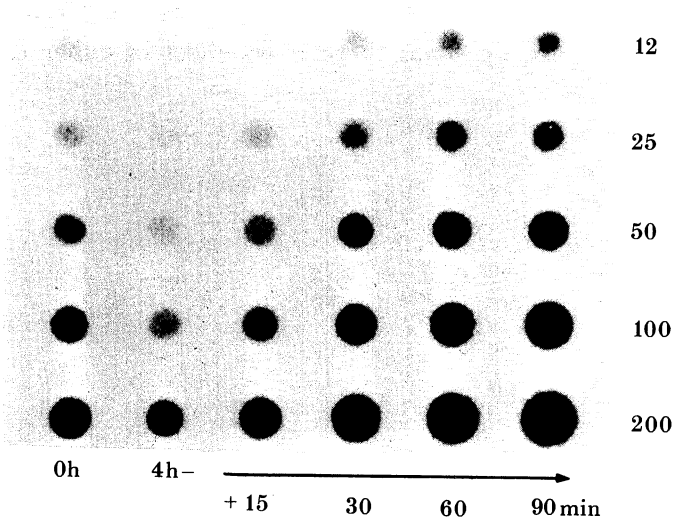


FIGURE 5. Rapid accumulation of an auxin-regulated mRNA during auxin-induced cell elongation. Poly(A)$^+$ RNA from excised elongating sections was bound to nitrocellulose and probed with pJCW2. Concentrations from top to bottom are 12, 25, 50, 100 and 200 ng of poly(A)$^+$ RNA. RNA was isolated from tissue that was unincubated or tissue incubated without auxin for 4 h, or 4 h without auxin, followed by auxin treatment for 15, 30, 60 and 90 min. (Data of J. Walker and J. L. Key.)

Several lines of evidence strongly suggest that the change in relative abundance of the JCW1 and JCW2 RNAs is in fact an auxin-dependent response (Walker *et al.* 1985). First, the sequences increase similarly in response to several active auxins, but not to inactive related compounds. Second, the change in concentration of these poly(A)$^+$ RNAs is not simply a response to changes in the rate of cell elongation of the tissue: (*a*) fusicoccin increases the rate

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

of cell elongation in tissue incubated without auxin to rates above the maximum induced by auxin, but the JCW1 and JCW2 RNAs remain at levels similar to that of the auxin-free medium where cell elongation is minimal; and (*b*) cytokinin significantly inhibits auxin-induced growth in excised soybean hypocotyl but does not affect the auxin-induced accumulation of JCW1 and JCW2 RNAs. Hanson & Trewavas (1982) showed that fusicoccin-induced cell elongation is not dependent on RNA and protein synthesis. They speculated that fusicoccin somehow bypasses the processes that require RNA and protein synthesis for auxin-stimulated cell elongation. In contrast to auxin-induced cell elongation, fusicoccin-induced cell elongation is not inhibited by cytokinin (Walker *et al.* 1985). If it is assumed that the auxin-enhanced rate of cell elongation is the result of auxin-enhanced accumulation (synthesis) of specific 'rate-limiting' mRNAs and their translation products, the action of cytokinin in inhibiting auxin-induced cell elongation (but not fusicoccin-induced cell elongation) may well relate to the inhibition of synthesis of the 'rate-limiting' protein(s) or the inhibition of the function(s) of such protein(s). Since cytokinin does not affect the accumulation of these RNAs, either their protein products are not a part of the rate limiting step(s) of cell elongation or cytokinin inhibition of auxin-induced elongation occurs at the level of protein synthesis or function. Cytokinin does in fact inhibit protein synthesis in the excised soybean hypocotyl tissue (see, for example, Zurfluh & Guilfoyle 1980).

Since auxin treatment of tissue may induce ethylene production, an apparent auxin response can be either a direct response to auxin or an indirect response mediated by ethylene. Walker *et al.* (1985) showed that ethylene has no effect on JCW1 and JCW2 RNAs alone or in combination with auxin. A precursor of ethylene, ACC, and an inhibitor of ethylene biosynthesis, AVG, have no effect on the expression of JCW1 and JCW2 RNAs in the presence or absence of auxin (figure 6). Thus there is no effect of either exogenously applied or

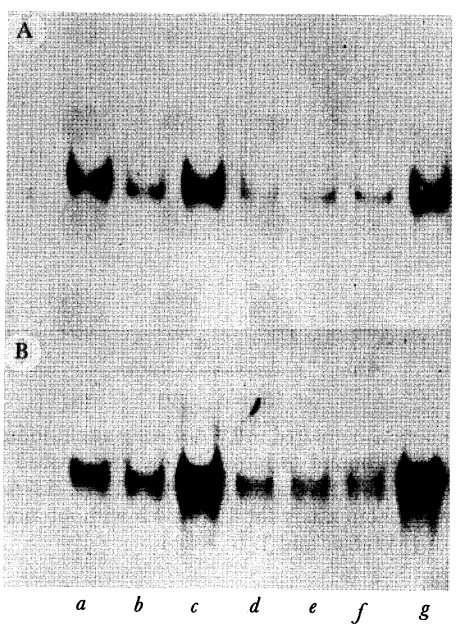


FIGURE 6. RNA blot hybridization analysis of poly(A)$^+$ RNA hybridizing to auxin-responsive cDNA clones pJCW1 or pJCW2. Poly(A)$^+$ RNA (0.5 μg) was isolated from elongating tissue and fractionated by electrophoresis on a 6% formaldehyde–2% agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labelled pJCW1 (A) or pJCW2 (B). RNA was isolated from elongating sections incubated for 2 h without hormone (*b*), or incubated for 2 h without hormone followed by a 4 h incubation with (*c*) 2,4-D, (*d*) ACC, (*e*) AVG, (*f*) AVG+ACC or (*g*) 2,4-D+AVG. RNA from unincubated sections is shown in (*a*). (From Walker & Key (1985).)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

endogenously produced ethylene on the concentration of these two auxin-response mRNAs, and ethylene does not alter the influence of auxin on their accumulation.

In hybrid-selection translation experiments (J. Walker & J. L. Key, unpublished data), pJCW1 hybrid-selected poly(A)$^+$ RNAs that translated into polypeptides of 31 and 30 kDa with p*I*s of about 6.1 and 6.7, respectively. Clone pJCW2 hybrid-selected mRNAs encoding a major polypeptide of 22 kDa on 1D SDS gels and a minor polypeptide of 19 kDa. The 22 kDa polypeptide was resolved in O'Farrell 2D gels into five polypeptides with p*I*s ranging from 5.9 to 7.2; the 19 kDa polypeptide(s) did not focus on the O'Farrell system.

Clones to IAA-induced mRNAs were recently isolated from pea epicotyl tissue undergoing enhanced rates of cell elongation in response to auxin (Theologis & Ray 1982; Theologis *et al.* 1985). One major difference in the induction of these mRNAs in pea epicotyl from those induced by auxin in soybean hypocotyl relates to the effects of cycloheximide on their production. Cycloheximide has no major effect either in the presence or absence of auxin on the accumulation of the soybean hypocotyl poly(A)$^+$ RNAs noted above. However, cycloheximide alone induces the pea epicotyl RNAs described by Theologis *et al.* (1985). It is unknown whether the proteins encoded by the mRNAs homologous to these three sets of cDNAs have any common functions. Any relations (homologies) among these different cloned sequences will be defined from DNA sequence analysis of the cDNAs, corresponding genomic clones, and/or the derived amino acid sequence of the encoded proteins. It is known that the cDNA sequences isolated by Hagen *et al.* (1984) have no significant homologies to those of Walker & Key (1982) based on a lack of cross-hybridization. However, the lack of significant cross-hybridization (and even nucleotide sequence homology) between pJCW1 and pJCW2 does not reflect the very significant homologies at the amino acid level (see below).

Isolation of genomic clones to auxin-regulated poly(A)$^+$ RNAs

Genomic clones have been isolated that are homologous to pJCW1 and pJCW2 (Walker 1985), each of the three sequences isolated by Baulcombe & Key (1980) which are down-regulated by auxin (P. Kroner & R. T. Nagao, unpublished data), and several of the r-protein cDNAs isolated by Gantt & Key (1985). Sequence analysis and nuclease (S_1 or mung bean) mapping of the 5′ and 3′ ends of the corresponding mRNA have been completed on some of these genes as a first step towards determining the mechanism by which auxin regulates their expression and the function of the encoded proteins. As an example, genes homologous to pJCW1 and pJCW2 have five and three introns, respectively (figure 7). The 5′ end of the mRNA corresponding to pJCW2 maps about 33 base pairs downstream of a TATA box sequence. Rather typical of many plant genes sequenced to date, neither gene contains a consensus polyadenylation signal, AATAAA, found in many genes of higher eukaryotes. Each gene is a member of a small multigene family. The sequence analyses revealed several interesting features of the JCW1 and JCW2 proteins (J. Walker & J. L. Key, unpublished data). Both are very hydrophilic proteins with very similar hydropathic profiles (Kyte & Doolittle 1982). Both proteins have potential glycosylation sites, but the absence of a signal peptide in either protein would suggest that these may not be functional sites for glycosylation. A search of the Protein Identification Resource protein sequence data bank has revealed no significant homologies with the derived amino acid sequence of these proteins and the sequences of other published proteins. Although there are no continuous nucleotide homologies of more than 11 nucleotides in the cDNAs (or in the genomic sequences), there are five highly conserved

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

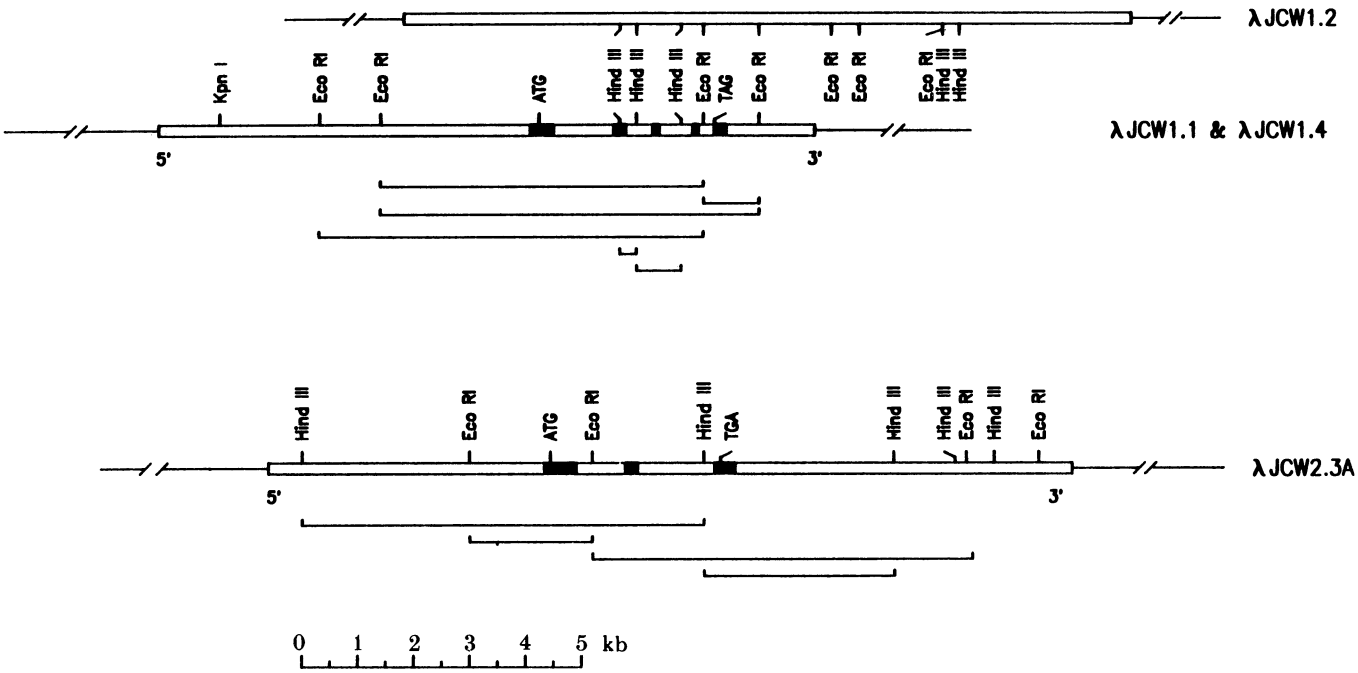


FIGURE 7. Restriction endonuclease maps of the recombinant phage containing genomic DNA hybridizing to pJCW1 (λJCW1.1, λJCW1.2, λJCW1.4) or pJCW2 (λJCW2.3A). The open boxes illustrate the genomic DNA and the solid boxes represent the exon regions of the genes. The brackets below the genomic clones illustrate the restriction fragments used for subcloning and sequence analysis. The direction of transcription as shown is 5′→3′. (From Walker (1985).)

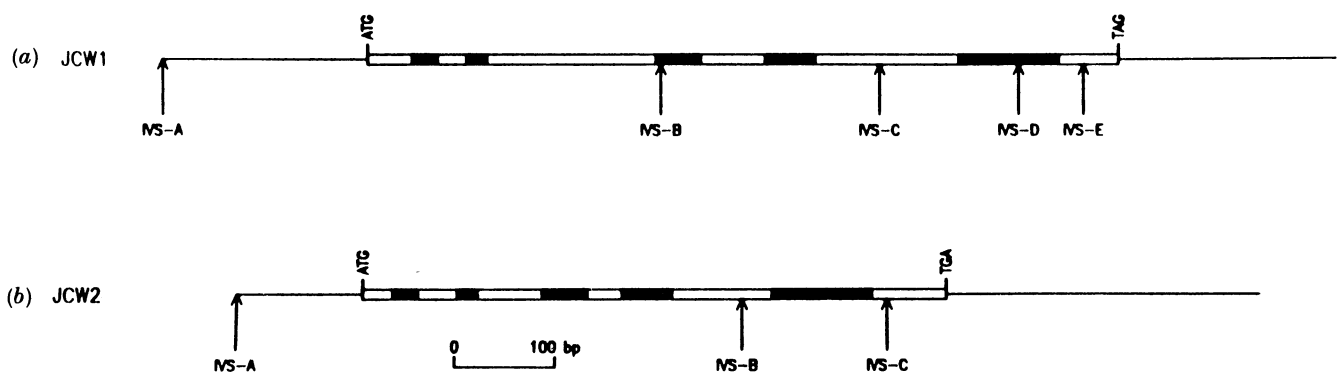


FIGURE 8. Intron positions relative to the coding sequence. The positions of the introns (IVS) in the JCW1 (*a*) and JCW2 (*b*) genes are illustrated by the vertical arrows. The horizontal bars indicate the coding sequence and the horizontal lines the 5′ and 3′ non-translated regions. The solid bars represent regions encoding amino acids conserved between the two deduced protein sequences.

(80–100%) regions of amino acid sequence homology consisting of 7, 8, 15, 17 and 33 amino acids in the proteins encoded by JCW1 and JCW2 RNAs (figure 8). These regions of conservation seem to have little or no relation to the intron positions in the corresponding genes.

ANALYSIS OF A DEVELOPMENTALLY (AUXIN?) REGULATED GENE

As a follow-up to studies on poly(A)$^+$ RNA changes in cultured soybean cells during growth-rate shifts associated with altered levels of hormones in the growth medium (T. H. Ulrich & J. L. Key, unpublished data), a cDNA library was made to poly(A)$^+$ RNA isolated from those

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

cells. A cDNA clone was isolated that appeared to be an auxin-responsive sequence. The poly(A)$^+$ RNA was present at low levels in auxin-depleted cells and at higher levels in medium supplemented with auxin. However, elimination of both auxin and cytokinin from the medium resulted in greatly increased levels of this poly(A)$^+$ RNA, with the major increase occurring in a larger poly(A)$^+$ RNA (about 1220, compared with 1050, nucleotides in length). Northern blot analyses were made with the use of this cDNA clone (designated pTU04) to probe poly(A)$^+$ RNAs from different regions of the soybean hypocotyl. Results of these experiments demonstrated that this cloned sequence hybridized to an RNA of about 1050 nucleotides in length from the apical and elongating actively growing zones and that the major hybridizing RNA of mature hypocotyl was about 1220 nucleotides long (figure 9). Some RNA 1050 nucleotides long was present in mature tissue with low levels of an additional RNA 650 nucleotides long. Thus there appears to be a definite developmental switch in size of mRNA that hybridizes to pTU04 during maturation of soybean hypocotyl cells. Auxin treatment of the hypocotyl induces a shift in message size, with some of the 1220 larger form being produced in the apical hypocotyl tissue associated with a considerable increase in the level of the smaller 1050 form in the mature region (unpublished data of T. H. Ulrich, P. Kroner & J. L. Key) consistent with the effects of auxin on the growth patterns of these tissues (Key *et al.* 1966). Thus meristematic and actively growing cells appear to produce primarily the smaller form of this poly(A)$^+$ RNA whereas relatively quiescent tissues produce primarily the larger form of poly(A)$^+$ RNA hybridizing to pTU04.

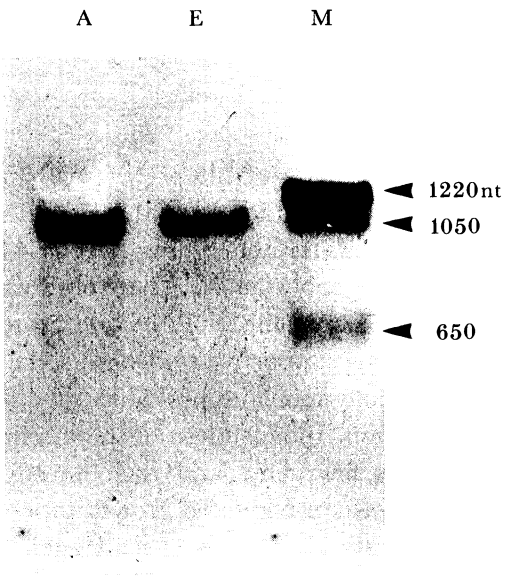


FIGURE 9. Northern blot analysis of poly(A)$^+$ RNA from soybean hypocotyl: 1 μg of poly(A)$^+$ RNA isolated from apical (A), elongating (E) and mature (M) zones was separated by electrophoresis on 6% formaldehyde–2% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labelled pTU04. The size of RNA hybridizing to pTU04 was determined by using *Alu*I-digested pBR322 as standards. (From Hong *et al.*, in preparation.)

As one approach to assessing the mechanism of this developmental switch, genomic clones having homology to pTU04 have been isolated and are being analysed, including by DNA sequence analysis. One clone has been sequenced (J. C. Hong, R. T. Nagao & J. L. Key, in preparation) that has complete nucleotide homology in the coding region to the pTU04 cDNA. This gene produces the large form poly(A)$^+$ RNA, which codes for a protein of 256 amino acid

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

residues with a calculated molecular mass of 29 kDa; a second open reading frame, which would code for a shorter protein (22 kDa), occurs on the same strand. The hybrid select-translated protein appears to have a molecular mass of about 30 kDa and an estimated p*I* of about 10, suggesting that the first open reading frame is in fact the translated reading frame.

Analysis of this gene indicates that there is no obvious way to produce the 1050 and 650 nucleotide forms of pTU04-hybridizing poly(A)$^+$ RNA from this DNA sequence, suggesting of the presence of different but related genes (a suggestion confirmed by direct analysis (J. C. Hong & R. T. Nagao, unpublished data)) that are differentially expressed during development. The gene has no introns, and codes for a protein having properties expected of a cell-wall protein (Chen & Varner 1985*a*, *b*). There is a presumptive 26 amino acid leader or signal sequence followed by 45 near-repeats of Pro-Pro-Val-Tyr-Lys. This sequence is clearly different from the extensin gene sequenced from carrot (Chen & Varner 1985*a*) but possibly related to a second 33 kDa proline-rich protein cDNA sequence that has been characterized from carrot (Chen & Varner 1985*b*).

It should be emphasized that it has not been established that the TU04 gene encodes a cell-wall protein. However, similarities in structure between the derived amino acid sequence with other known cell-wall proteins strongly suggest that the TU04 gene encodes a protein destined for the cell wall. Future research will involve the characterization of proline-rich proteins (or more likely hydroxyproline-rich glycoproteins) in the cell wall of soybean hypocotyl. Although additional information is needed, data presented here are suggestive of a role of TU04-related proteins in cell differentiation and maturation and that alterations in expression of genes for putative cell-wall proteins by auxin may be important in these developmental transitions.

Summary and conclusions

Definitive evidence has been provided for a rapid, dramatic and specific effect of auxin on the expression of a few genes in tissues undergoing both cell division and cell elongation in response to the application of exogenous auxin. These observations, coupled with the earlier work that showed an absolute requirement for RNA and protein synthesis for cell elongation in response to auxin, provide strong supportive evidence for the concept that auxin 'responses' in controlling cell division, cell elongation and probably cell differentiation are tightly coupled to auxin-regulated gene expression. Independent of the mechanism(s) of auxin 'action', the auxin-regulated expression of a few genes and their encoded proteins must be of major importance in the physiology of auxin-regulated growth processes.

Nuclear run-off transcription studies (figure 4; see Hagen & Guilfoyle (1985) for details) indicate that auxin treatment results in a very rapid induction of transcription of those RNA sequences that are responsive to the hormone. Moreover, several auxin-regulated mRNAs have been shown to be induced within 5–30 min in tissue incubated with auxin. It is tempting to speculate that some of these rapid changes in poly(A)$^+$ RNA level may represent primary responses to auxin. These changes certainly are induced in the same time frame that steroid hormone-induced synthesis of various mRNAs occurs in responsive tissues. The induction or enhancement of transcription of responsive genes by steroid hormones generally results from the interaction of the steroid hormone receptor with gene sequences (and/or chromatin structures) 5′ to the transcription start sites of these genes. Even in these elegant studies that

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

are the culmination of work done over many decades and with the enormous progress made over the last several years in understanding steroid hormone-regulated gene expression, there remain many unknowns about the details of these systems. Although the results on steroid hormone-regulated gene expression may serve as one of several models for the mechanism underlying auxin-regulated gene expression, so far there is no information available to indicate whether such a mechanism will apply to the auxin response. A series of studies designed to define *cis*-acting elements important to the regulated expression of the auxin-responsive genes are continuing. These analyses will be coupled with experiments designed to define *trans*-acting elements (or transcription factors) involved in the regulated expression of these genes. A significant question relates to whether auxin/'auxin receptors' are a part of this transcription regulatory system (see Cross (1985) and Lobler & Klambt (1985*a*, *b*) for discussion of auxin receptors). Although considerable effort has been devoted to auxin-binding proteins and transcription, little, if any, understanding has been gained from these studies about auxin regulation of gene expression (see Guilfoyle 1986). Thus a considerable research effort is needed to gain insight into the mechanism(s) by which auxin modulates gene expression.

Current research on auxin-regulated gene expression is supported by grant GM30317 from the NIH. Research contributions by many colleagues is most appreciated. The assistance of Joyce Kochert and Ginger Vickery in manuscript preparation is acknowledged. The authors are most appreciative to Dr Gretchen Hagen and Dr Tom Guilfoyle for permission to use data (figures 3 and 4) from their recent work in this brief review.

References

Baulcombe, D., Giorgini, J. & Key, J. L. 1980 The effect of auxin on the polyadenylated RNA of soybean hypocotyls. In *Genome organization and expression in plants* (ed. C. J. Leaver), pp. 175–185. New York: Plenum Press.

Baulcombe, D. C. & Key, J. L. 1980 Polyadenylated RNA sequences which are reduced in concentration following auxin treatment of soybean hypocotyls. *J. biol. Chem.* **255**, 8907–8913.

Baulcombe, D. C., Kroner, P. A. & Key, J. L. 1981 Auxin and gene regulation. In *Levels of genetic control in development* (ed. Subtelny & U. K. Abbott), pp. 83–97. New York: Alan R. Liss.

Chen, J. & Varner, J. E. 1985*a* An extracellular matrix protein in plants: characterization of a genomic clone for carrot extensin. *EMBO J.* **4**, 2145–2151.

Chen, J. & Varner, J. E. 1985*b* Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4399–4403.

Cross, J. W. 1985 Auxin action: the search for the receptor. *Plant Cell Envir.* **8**, 351–359.

Evans, M. L. 1985 The action on plant cell elongation. *CRC crit. Rev. Pl. Sci.* **2**, 317–365.

Evans, M. L. & Ray, P. M. 1969 Timing of the auxin response in coleoptiles and its implications regarding auxin action. *J. gen. Physiol.* **53**, 1–20.

Gantt, J. S. & Key, J. L. 1985 Coordinate expression of ribosomal protein mRNAs following auxin treatment of soybean hypocotyls. *J. biol. Chem.* **260**, 6175–6181.

Guilfoyle, T. J. 1986 Auxin regulated gene expression in higher plants. *CRC crit. Rev. Pl. Sci.* (In the press.)

Guilfoyle, T. J., Lin, C.-Y., Chen, Y.-M., Nagao, R. T. & Key, J. L. 1975 Enhancement of soybean RNA polymerase I by auxin. *Proc. natn. Acad. Sci. U.S.A.* **72**, 69–72.

Hagen, G. & Guilfoyle, T. J. 1985 Rapid induction of selective transcription by auxins. *Molec. Cell Biol.* **5**, 1197–1203.

Hagen, G., Kleinschmidt, A. & Guilfoyle, T. 1984 Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. *Planta* **162**, 147–153.

Hanson, J. B. & Slife, F. W. 1969 Role of RNA metabolism in the action of auxin herbicides. *Residue Rev.* **25**, 59–67.

Hanson, J. B. & Trewavas, A. J. 1982 Regulation of plant cell growth: the changing perspective. *New Phytol.* **90**, 1–18.

Key, J. L. & Ingle, J. 1964 Requirement for the synthesis of DNA-like RNA for growth of excised plant tissue. *Proc. natn. Acad. Sci. U.S.A.* **52**, 1382–1388.

Key, J. L. & Shannon, J. C. 1964 Enhancement by auxin of ribonucleic acid synthesis in excised soybean hypocotyl tissue. *Pl. Physiol.* **39**, 360–364.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Key, J. L. 1964 Ribonucleic acid and protein synthesis as essential processes for cell elongation. *Pl. Physiol.* **39**, 365–370.

Key, J. L., Lin, C.-Y., Gifford, E. M. Jr & Dengler, R. 1966 Relation of 2,4-D-induced growth aberrations to changes in nucleic acid metabolism in soybean seedlings. *Bot. Gaz.* **127**, 87–94.

Key, J. L., Barnett, N. M. & Lin, C.-Y. 1967 RNA and protein biosynthesis and the regulation of cell elongation by auxin. In *Ann. N.Y. Acad. Sci.* **144**, 49–62.

Key, J. L. & Ingle, J. 1968 RNA metabolism in response to auxin. In *Biochemistry and physiology of plant growth substances* (ed. F. Wightman & G. Setterfield), pp. 711–722. Ottawa: Runge Press.

Key, J. L. 1969 Hormones and nucleic acid metabolism. *A. Rev. Pl. Physiol.* **20**, 449–474.

Kyte, J. & Doolittle, R. F. 1982 A simple method for displaying the hydropathic character of a protein. *J. molec. Biol.* **157**, 105–132.

Lobler, M. & Klambt, D. 1985*a* Auxin-binding protein from coleoptile membranes of corn (*Zea mays*. L.). I. Purification by immunological methods and characterization. *J. biol. Chem.* **260**, 9848–9853.

Lobler, M. & Klambt, D. 1985*b* Auxin-binding protein from coleoptile membranes of corn (*Zea mays*. L.). II. Localization of a putative auxin receptor. *J. biol. Chem.* **260**, 9854–9859.

Skoog, F. 1954 Substances involved in normal growth and differentiation in plants. *Brookhaven Symp. Biol.* **6**, 1–21.

Terry, M. E. & Jones, R. L. 1981 Effect of salt on auxin-induced acidification and growth by pea internode sections. *Pl. Physiol.* **68**, 59–64.

Theologis, A. & Ray, P. M. 1982 Early auxin-regulated polyadenylated mRNA sequences in pea stem tissue. *Proc. natn. Acad. Sci. U.S.A.* **79**, 418–421.

Theologis, A., Huynh, T. V. & Davis, R. W. 1985 Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *J. molec. Biol.* **183**, 53–68.

Trewavas, A. 1968 Relationship between plant growth hormones and nucleic acid metabolism. *Prog. Phytochem.* **1**, 113–161.

Vanderhoef, L. N. 1980 Auxin-regulated cell enlargement: is there action at the level of gene expression? In *Genome organization and expression in plants* (ed. C. J. Leaver), pp. 159–173. New York: Plenum Press.

Walker, J. C. 1985 Auxin regulation of gene expression. Ph.D. thesis, University of Georgia.

Walker, J. C. & Key, J. L. 1982 Isolation of cloned cDNAs to auxin-responsive poly(A)$^+$ RNAs of elongating soybean hypocotyl. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7185–7189.

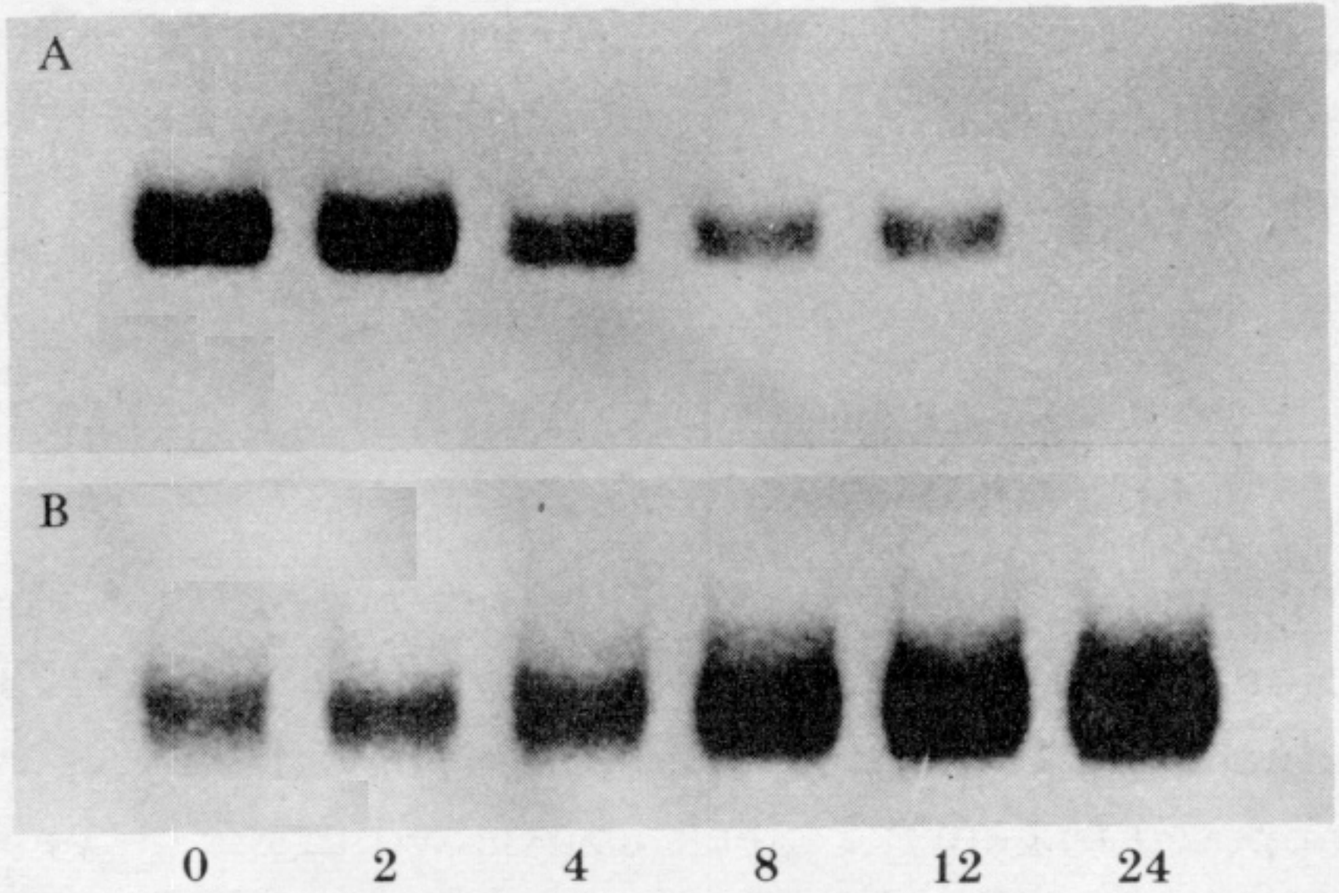
Walker, J. C., Legocka, J., Edelman, L. & Key, J. L. 1985 An analysis of growth regulator interactions and gene expression during auxin-induced cell elongation using cloned complementary DNAs to auxin-responsive messenger RNAs. *Pl. Physiol.* **77**, 847–850.

Zurfluh, L. L. & Guilfoyle, T. J. 1980 Auxin-induced changes in the patterns of protein synthesis in soybean hypocotyl. *Proc. natn. Acad. Sci. U.S.A.* **77**, 357–361.

Zurfluh, L. L. & Guilfoyle, T. J. 1982*a* Auxin-induced changes in the population of translatable messenger RNA in elongating sections of soybean hypocotyl. *Pl. Physiol.* **69**, 332–337.

Zurfluh, L. L. & Guilfoyle, T, J. 1982*b* Auxin- and ethylene-induced changes in the population of translatable messenger RNA in basal sections and intact soybean hypocotyl. *Pl. Physiol.* **69**, 338–340.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



GURE 1. Effect of auxin on the concentration of two soybean mRNAs. Northern analysis of poly(A)$^+$ RNA isolated from auxin-treated 4-day-old etiolated mature hypocotyl. The blots were probed with cDNA inserts labelled by nick-translation. A, Probed with cDNA p11 (Baulcombe & Key 1980); B, probed with cDNA encoding cytoplasmic ribosomal protein L13 (Gantt & Key 1985); numbers indicate time (hours) of auxin treatment, respectively. (Data of P. Kroner & J. L. Key.)

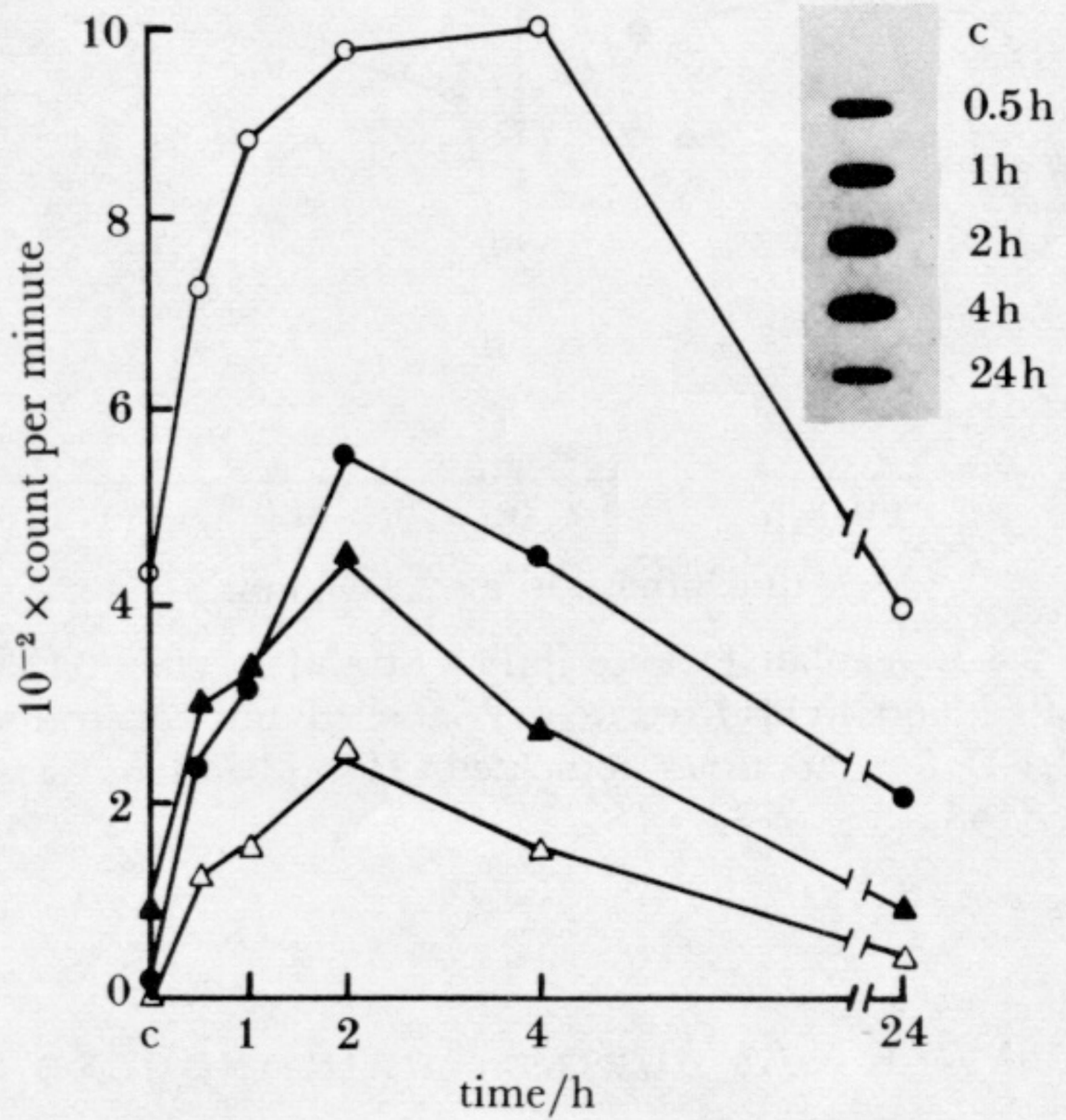


FIGURE 3. Hybridization analysis of cDNA plasmids to RNA from untreated (c) and 2,4-D-treated intact soybean hypocotyl. After treatment of the hypocotyl with 2,4-D (for 0.5, 1, 2, 4 or 24 h), poly(A)$^+$ RNA was extracted; 2 μg of RNA was denatured, 'slot-blotted' onto nitrocellulose and hybridized to labelled cDNA plasmids. The autoradiogram of the hybridization of pGH3 to the slot-blot is shown (inset). Radioactivity associated with individual slots was determined by scintillation counting and the activity (the average of duplicate slots) plotted (○, pGH1; △, pGH2; ●, pGH3; ▲, pGH4). (From Hagen *et al.* (1984).)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

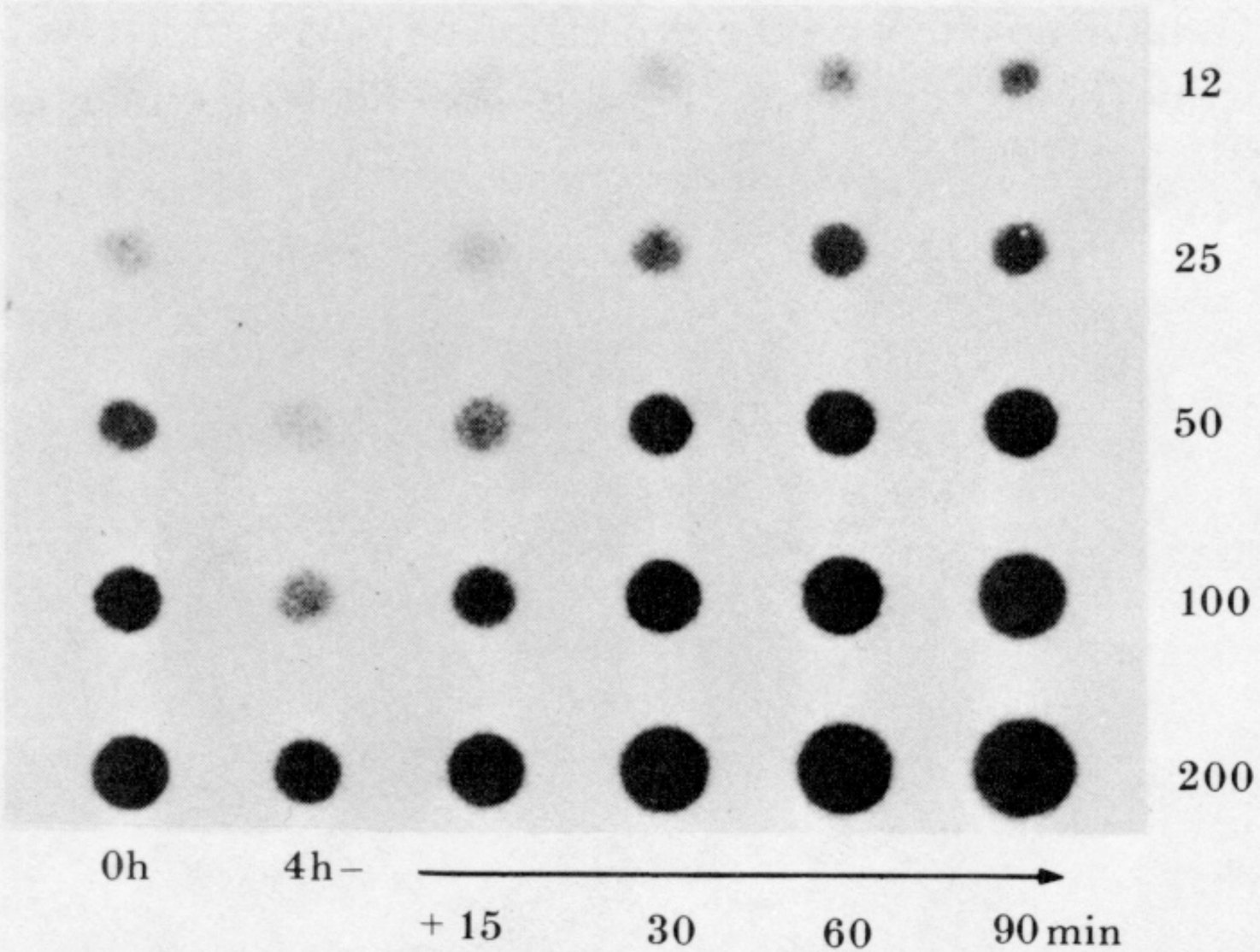


FIGURE 5. Rapid accumulation of an auxin-regulated mRNA during auxin-induced cell elongation. Poly(A)$^+$ RNA from excised elongating sections was bound to nitrocellulose and probed with pJCW2. Concentrations from top to bottom are 12, 25, 50, 100 and 200 ng of poly(A)$^+$ RNA. RNA was isolated from tissue that was unincubated or tissue incubated without auxin for 4 h, or 4 h without auxin, followed by auxin treatment for 15, 30, 60 and 90 min. (Data of J. Walker and J. L. Key.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

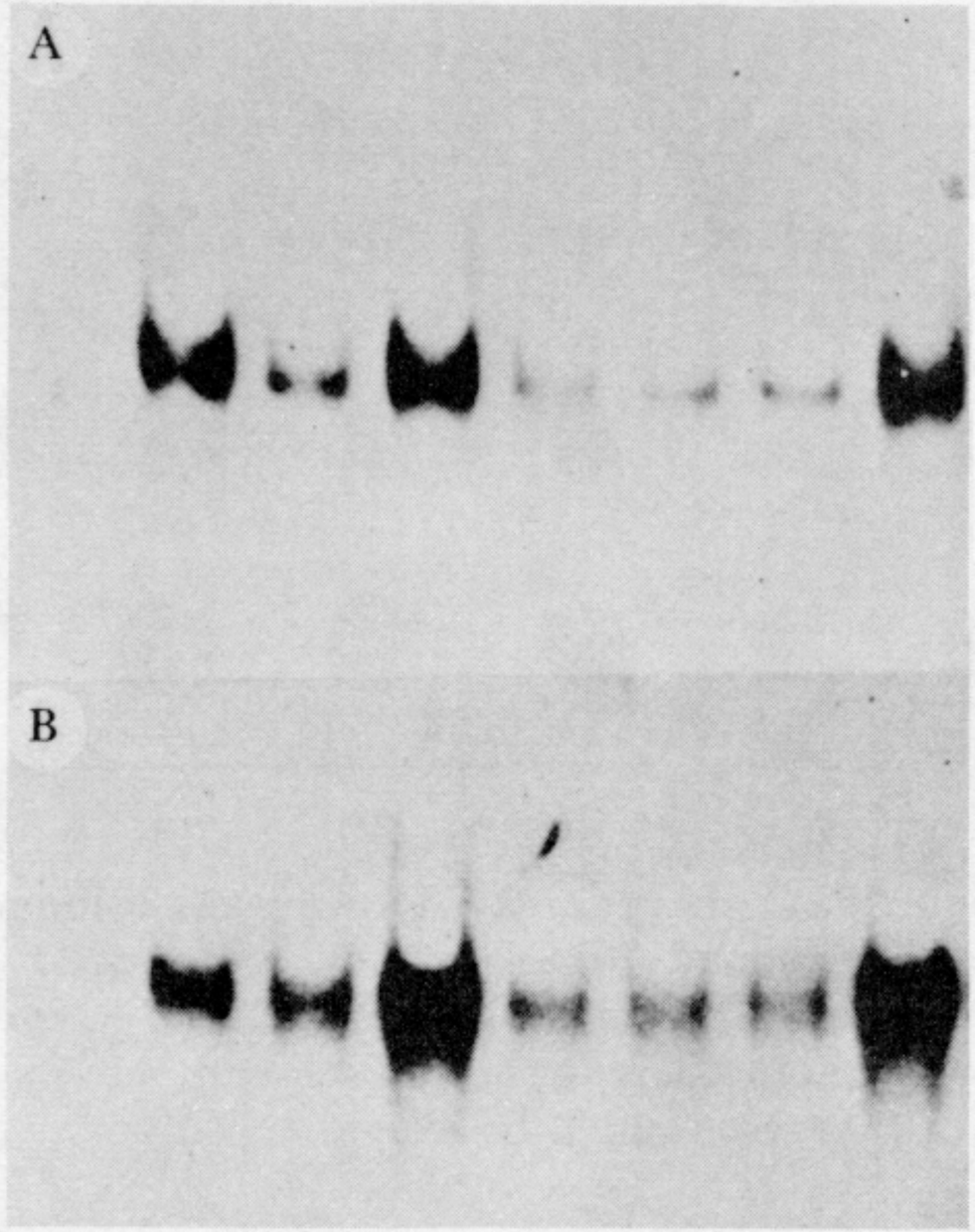
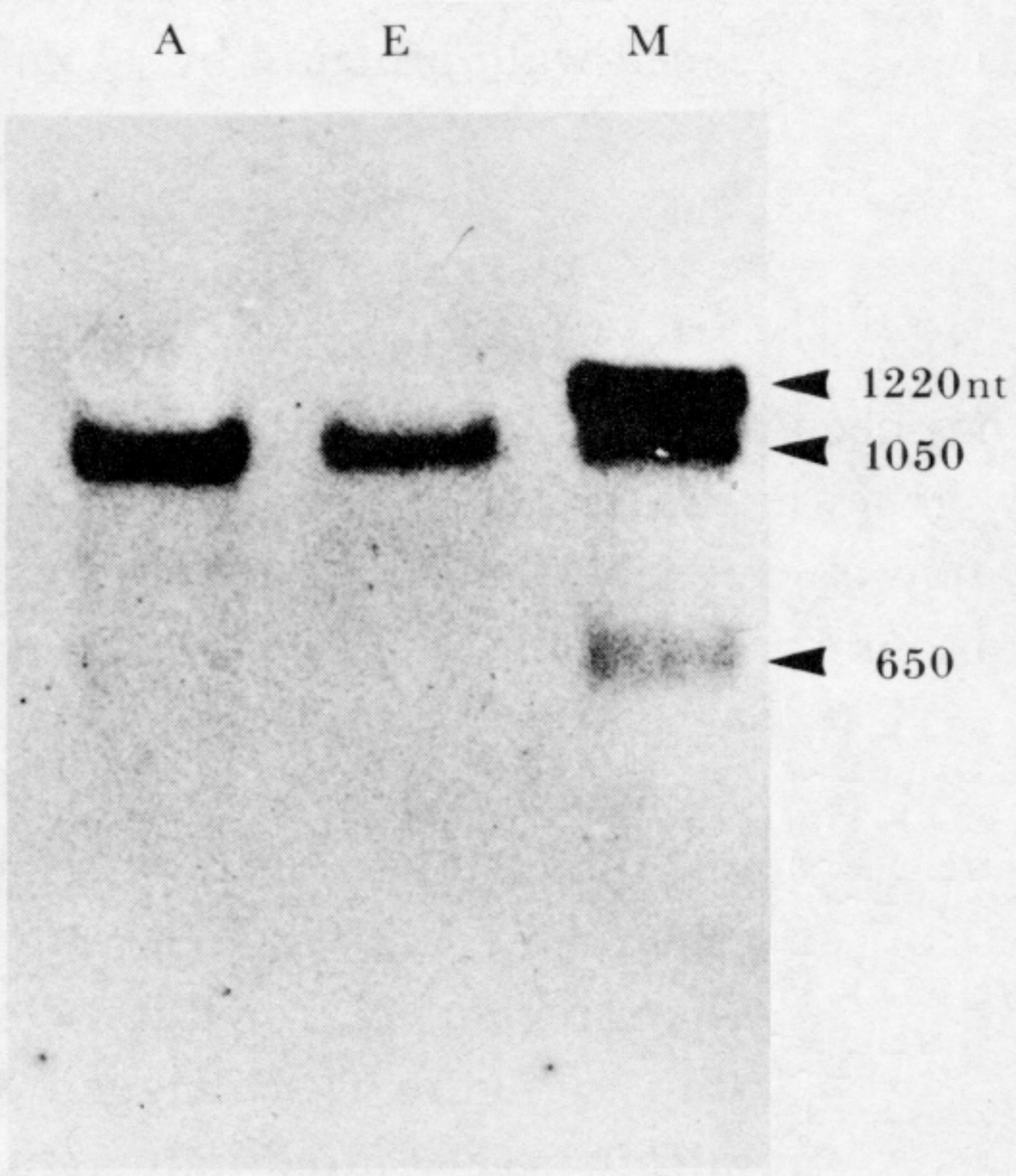


FIGURE 6. RNA blot hybridization analysis of poly(A)$^+$ RNA hybridizing to auxin-responsive cDNA clones pJCW1 or pJCW2. Poly(A)$^+$ RNA (0.5 μg) was isolated from elongating tissue and fractionated by electrophoresis on a 6% formaldehyde–2% agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labelled pJCW1 (A) or pJCW2 (B). RNA was isolated from elongating sections incubated for 2 h without hormone (*b*), or incubated for 2 h without hormone followed by a 4 h incubation with (*c*) 2,4-D, (*d*) ACC, (*e*) AVG, (*f*) AVG+ACC or (*g*) 2,4-D+AVG. RNA from unincubated sections is shown in (*a*). (From Walker & Key (1985).)



PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

GURE 9. Northern blot analysis of poly(A)$^+$ RNA from soybean hypocotyl: 1 μg of poly(A)$^+$ RNA isolated from apical (A), elongating (E) and mature (M) zones was separated by electrophoresis on 6% formaldehyde–2% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labelled pTU04. The size of RNA hybridizing to pTU04 was determined by using *Alu*I-digested pBR322 as standards. (From Hong *et al.*, in preparation.)