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An auxin-induced polypeptide in dicotyledonous plants

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Abstract

Antisera were raised to a 70-kD (kilodalton) soybean (*Glycine max*) protein encoded by a 2,4-dichlorophenoxyacetic acid (2,4-D) inducible mRNA, GH3. These antisera have been used to probe protein blots to study the kinetics and specificity of the GH3 induction response as well as the species specificity and intracellular location of the protein. Detectable levels of the GH3 protein are induced by 2,4-D within 2 h in elongating hypocotyl sections, root sections, and etiolated plumules, and within 30–60 min in soybean suspension cells. Synthesis of the GH3 protein is induced by a variety of auxins. Other plant hormones such as gibberellic acid, cytokinin and ethylene added with or whithout 2,4-D do not alter the level of GH3 protein induction. The GH3 protein is found only in the S100 fraction and is not associated with the nucleus or cell wall. This antiserum also reacts with a 2,4-D-inducible 70-kD protein in other dicots.

Introduction

The auxins are a group of plant hormones that dramatically affect the growth and development of plants. To determine if auxins function, in part, by affecting gene expression, investigators have compared the *in vitro* translation products produced by mRNA from auxin-treated and untreated tissues [1, 4, 17, 18, 26, 27, 28]. These studies showed that auxins were capable of stimulating or inhibiting the synthesis of specific mRNAs that code for polypeptides. Some of the polypeptide changes did not occur until more than 2 h after the addition of auxin to the tissues and are considered to be "late" mRNA changes. Several of these "late" mRNAs have been shown to code for polypeptides associated with the large and small ribosomal subunits [4]. Additionally, RNA polymerase I [5], cellulase [23], and 1,3-β-D-glucanase [25] increase upon auxin incubation and could be considered "late" auxin-iducible proteins.

Other *in vitro* translation differences have been detected as rapidly as 10–30 min after auxin addition [17, 18, 26, 27, 28]. These are considered to be "rapid" auxin responses. Several cDNAs have now been cloned that hybridize to rapidly auxin-inducible mRNAs [8, 16, 24]. Using these cDNAs, it has been shown that the increase in these specific mRNAs after auxin incubation is due, at least in part, to an increase in the transcription rates of these auxin-inducible genes [9, 16]. To understand the auxin responses, it will be necessary to study not only the genes, but also the gene products of these rapidly inducible genes. Antisera raised to these gene products might be useful for studying the kinetics of the protein induction response, for purifying the gene products and eventually determining the function of the gene products encoded by the auxin-responsive genes.

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We have raised polyclonal antisera to a fusion protein produced by inserting the auxin-inducible pGH3 cDNA [8] into an *E. coli* expression vector [7]. This antiserum reacts with a 70-kd protein in intact seedlings and excised 2,4-D incubated organs but not excised organs incubated without 2,4-D. Using this antiserum, we show that the root contains the highest and the dividing hypocotyl (hook) the lowest basal levels of GH3 protein in 3–14-d (day) soybean seedlings. A variety of auxins that induce the GH3 mRNA [9] also induce the GH3 protein. Other hormones such as gibberellic acid, cytokinin and ethylene affect neither the GH3 mRNA induction [9] nor the GH3 protein induction. An increase in the GH3 protein is detectable within 30–60 min after auxin addition to soybean suspension cells and within 2 h after auxin application to excised roots, elongating hypocotyls and plumules. The antiserum reacts with a protein of approximately 70 kd in other monocots and dicots we have tested.

Materials and methods

Plant material

Etiolated plants were grown in vermiculite-perlite (1 : 1) in darkness at 26 °C. Etiolated organ samples were from 5-d-old maize (*Zea mays*), 5-d-old mung bean (*Vigna radiata*), 3–8-d-old soybean (*Glycine max* var. Wayne), 4-d-old pea (*Pisum sativum* var. Alaska), 4-d-old green bean (*Phaseolus vulgaris* var. Blue Lake), and 4-d-old wheat (*Triticum aestivum*). Light-grown plants were maintained at 26 °C and a 12-h photoperiod. Green organ samples were from 60-d-old petunia (*Petunia hybrida* var. Mitchell Diploid) and 3–14-d-old soybean. Callus derived from excised elongating hypocotyl soybean sections was used to generate suspension cultures. The suspension cultures were maintained in the dark in Murashige and Skoog (MS) media [15] containing 10 μM 2,4-D, 1 μM cytokinin and 1 g/l casamino acids.

Organ incubations

Induction studies were done using 3-d-old excised soybean roots, 3-d-old elongating hypocotyl sections, 7-d-old etiolated plumules, or soybean suspension cultures. All excised organs were preincubated without 2,4-D for 8 h in incubation buffer (2% sucrose, 10 mM potassium phosphate (pH 6.0), 50 μg/ml chloramphenicol) prior to incubation with 100 μM 2,4-D in the incubation buffer. Organ-specific differences in basal levels of GH3 protein were assayed by excising organs from soybean plants grown for 3–7 d in darkness or 3–14 d with a 12-h photoperiod. the effects of gibberellic acid, cytokinin, and fusicoccin on GH3 protein induction were assessed by incubating soybean elongating sections with these compounds (100 μM) alone or with 2,4-D (100 μM) in incubation buffer for 6 h. The effect of ethylene on GH3 protein induction was tested by spraying 3-d-old intact soybean seedlings with the ethylene-generating compound Ethephon (1 mg/ml) with or without 100 μM 2,4-D and harvesting the hypocotyl sections 16 h later. Organs used for dose response experiments were excised, preincubated for 4 h without 2,4-D and then incubated in different concentrations of 2,4-D for 8 h.

Organelle isolation

Etiolated 7-d soybean plumules were incubated in 100 μM 2,4-D for 8 h. The plumules were then rinsed with distilled water and placed in ice-cold diethyl ether for 1 min and allowed to air dry for 5 min. An equal volume (w/v) of isolation buffer (10 μM Tris (pH 7.2), 5 mM $MgCl_2$, 1 M sucrose, 10 mM 2-mercaptoethanol) was added and the plumules were ground with a polytron for 1 min [9]. The homogenate was then filtered through Miracloth (Calbiochem) and centrifuged at 2000 *g* for 15 min to pellet nuclei and cell walls. The presence of intact nuclei was confirmed by light microscopy using azure C stain in 250 mM sucrose and 0.02% sodium azide. The supernatant was then centrifuged at 10000 *g* for 15 min. The resulting supernatant was then centrifuged at 100000 *g* for 3 h to pellet ribosomes.

Production of antisera

The soybean pGH3 cDNA [8] was cloned into the pWR 590 expression vector series [7] via Eco RI-Sal I ends. The β-galactosidase-GH3 fusion protein was overexpressed in *E. coli* strain MM294 and purified as described by Guo *et al.* [7]. The fusion protein was further purified by SDS-PAGE (polyacrylamide gel electrophoresis) [11] and visualized by Coomassie blue staining [3]. The fusion band was excised, pulverized and suspended in an equal volume of PBS (20 mM sodium phosphate (pH 7), 100 mM NaCl). Rabbits were immunized with a GH3-fusion/Freund's complete adjuvant (1:1) slurry and boosted 3 additional times at 2-wk intervals with a GH3-fusion/incomplete adjuvant (1 : 1) slurry and bled monthly. The IgG was purified as described by Linn *et al.* [12].

Sample preparation and protein blotting

After incubations, plant organs were frozen in liquid nitrogen and then ground to a powder with a mortar and pestle packed in dry ice. An aliquot of the ground tissue was weighed and 2 volumes (w/v) of sample buffer (10% glycerol, 3% SDS, 250 mM Tris (pH 6.8), 1 M 2-mercaptoethanol) was mixed into the sample. The samples were heated to 95 °C for 5 min, clarified by centrifugation for 1 min at 13000 *g* and subjected to SDS-PAGE. Polypeptides were then transferred to nitrocellulose (Schleicher and Schuell) according to Towbin *et al.* [19] using modifications of Guilfoyle *et al.* [6]. Pretreatment of the blot, incubation with antisera and washing were done as described by Guilfoyle *et al.* [6] with the following modifications. The antisera were diluted 1 : 1000 and incubated with the blot for 18–24 h, and the ^{125}I protein A (New England Nuclear, sp. act. 70 μCi/μg) was incubated with the blot for 4 h. Band intensities on autoradiograms were quantitated using a EC910 densitometer (E-C Apparatus Corp.).

To relate autoradiogram band intensities to the relative amount of GH3 protein present, increasing amounts of hypocotyl tissue were blotted and incubated with the GH3 antiserum and ^{125}I protein A. The resulting plot showed that binding of the GH3 antiserum and ^{125}I protein A was proportional to the amount of GH3 protein loaded. It also indicated the range of autoradiogram band intensity that the densitometer could accurately quantitate. Most of the bands quantitated using the densitometer were within the range of sensitivity. Those bands that appeared near the limit of sensitivity were subsequently cut out of the protein blots and quantitated by liquid scintillation counting. Signal intensities quantitated by scintillation counting agreed with those quantitated by densitometry.

Results

GH3 fusion protein and antisera

A 1200-bp (base pair) pGH3 cDNA was inserted into the frameshift polylinker of expression plasmids pWR590, pWR590-1 and pWR590-2 [7]. The new plasmid constructs contained a truncated β-galactosidase gene coding for a 75-kd polypeptide adjacent to the 1200-bp pGH3 cDNA inserted in all three reading frames. Plasmid pWR590-1 containing the pGH3 insert made a protein of 110 kd while pGH3 inserts in the pWR590 and pWR590-2 plasmids produced only the 75-kd β-galactosidase polypeptide as determined by SDS-PAGE (Fig. 1). The 110-kd molecular weight observed for the fusion protein agrees with the predicted molecular weight for a fusion between the β-galactosidase polypeptide and the polypeptide coded by the 1200-bp pGH3 cDNA.

Rabbits immunized with the GH3 fusion protein produced antisera that reacted with a 70-kd protein in untreated organs (not shown) or 2,4-D-treated elongating hypocotyl sections but not in sections preincubated in the incubation buffer (Fig. 2). RNA blot analysis has shown that the GH3 cDNA hybridizes to a 2400-bp mRNA [8] which is large enough to code for the 70-kd protein.

Intracellular location and organ specificity

Seven-day-old etiolated plumules were incubated in 100 μM 2,4-D, homogenized, and separated into fractions containing the cell walls and nuclei (2000 *g* pellet), a 10000 *g* pellet, ribosomes (100000 *g*

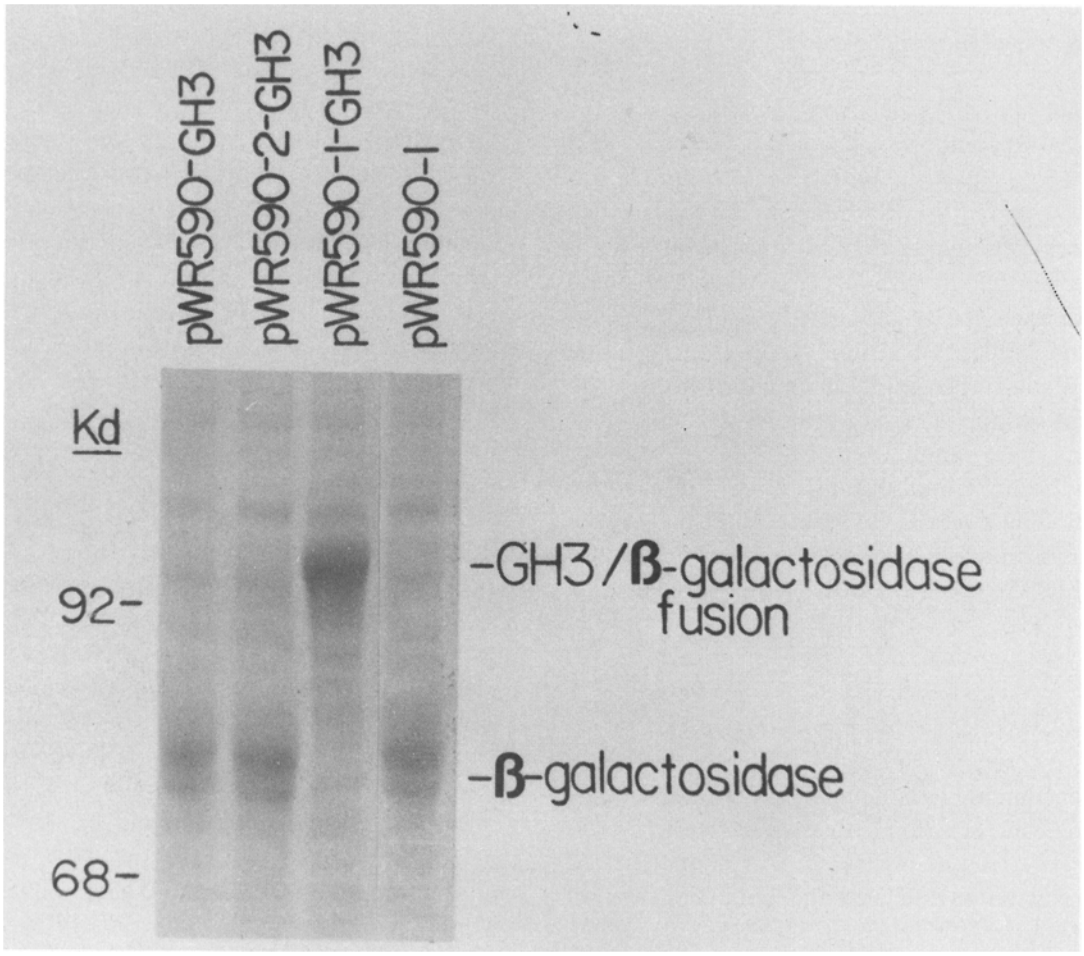

Fig. 1. Production of the β-gal-GH3 fusion protein in *E. coli*. Cell extracts from *E. coli*, strain MM294, transformed with pWR590-GH3, pWR590-2-GH3, pWR590-1-GH3 or pWR590-1 were subjected to SDS-PAGE and stained with Coomassie blue.

pellet), and 100000 *g* supernatant (S100). Equal amounts of protein from the fractions were analyzed by SDS-PAGE, transferred to nitrocellulose and incubated with GH3 specific antisera. Antibody binding indicates that the GH3 protein is most abundant in the cytoplasm (S100 fraction) and does not appear to be associated with the cell wall, nucleus, or other particulate fractions (Fig. 3). This subcellular location is the same in untreated and 2,4-D-treated tissue (data not shown).

The organ specificity of the GH3 protein was determined in 3-6–6-d and 8-d seedlings grown in the dark and 3-d–6-d, 8-d, 12-d, and 14-d seedlings grown with a 12-h photoperiod. Seedlings grown with a 12-h photoperiod showed equivalent organ-specific GH3 protein levels as those grown in the dark (data not shown). Figure 4 shows the basal (not induced by auxin) GH3 levels for a 4-d seedling grown in the dark. Roots showed the highest level of GH3 protein of the organs tested for all days analyzed. At 4–8 d the concentration of GH3 protein in the root was about 2-fold higher than mature hypocotyl sections, 5-fold higher than elongating hypocotyl sections and 7–10-fold higher than hook sections, or elongating epicotyl sections, plumules, or petioles (data not shown). However at 14 d the root sections are only 2–3-fold higher than mature and elongating hypocotyl sections or mature and elongating epicotyl sections. The 14-d leaf sections show almost no GH3 protein on blots; however, this

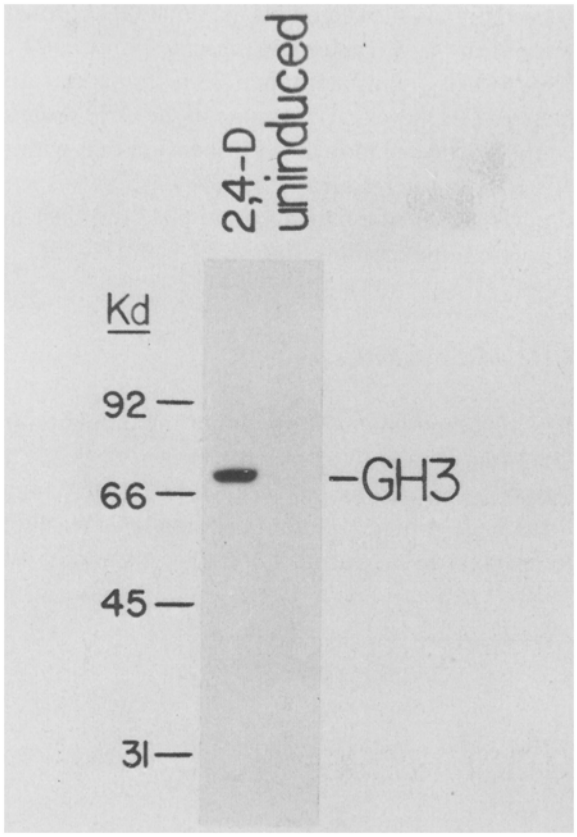

Fig. 2. Induction of the GH3-protein by 2,4-D. Excised 3-d soybean elongating hypocotyls were incubated for 8 h in incubation media alone or cotaining 100 μM 2,4-D. The samples were then frozen, ground in sample buffer, subjected to SDS-PAGE, transferred to nitrocellulose and incubated with GH3 antisera and ^{125}I protein A.

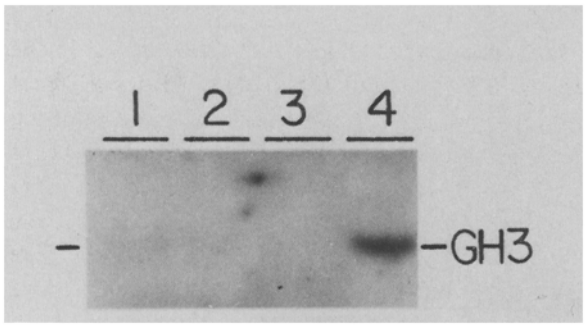

Fig. 3. Subcellular location of the GH3 protein. 7-d soybean plumules were fractionated as described in Materials and Methods. Equal amounts of protein from the fractions were subjected to SDS-PAGE, transferred to nitrocellulose and incubated with the GH3 antisera and ^{125}I protein A. Lane 1, 2000 *g* pellet; lane 2, 10000 *g* pellet; lane 3, 100000 *g* pellet; lane 4, 100000 *g* supernatant.

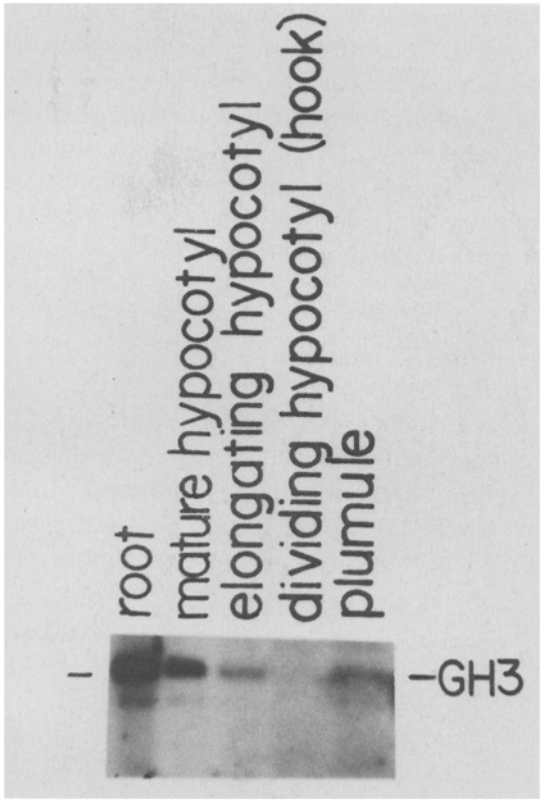

Fig. 4. Organ distribution of the GH3 protein. 5-d soybean seedlings were sectioned to separate the root, mature hypocotyl, elongating hypocotyl, hook, and plumule organs. The organs were frozen and treated as in Fig. 2.

may result from a gel artifact due to excessive amounts of ribulose-1,5-bisphosphate carboxylase large subunit migrating below the GH3 protein. Since roots contained the highest basal level of GH3 protein, mature root sections, root tip sections and lateral roots were excised from 4-d – 7-d old seedlings and assayed for GH3 protein. These root regions contained approximately equal amounts of the GH3 protein (data not shown).

Additional bands of higher and lower molecular weights were seen on the basal level blots. The lower molecular weight bands are thought to be proteins related to GH3 or breakdown products of the 70-kd GH3 protein, since they occur most often in lanes containing high levels of the GH3 protein. The higher molecular weight bands show up equally in

all tissues regardless of the amount of the GH3 protein present. These bands were not affected by the additions of auxins and may represent nonspecific binding resulting from antisera that were raised to an SDS-treated protein [2, 13]. However the possibility that these bands represent proteins that share homologous antigenic determinants with the GH3 protein has not been ruled out.

Kinetics of GH3 induction

The kinetics of GH3 induction in response to 2,4-D incubation were examined in excised roots, elongating hypocotyl sections, plumules, and soybean suspension cells. GH3 protein induction is detected within 2 h in hypocotyls, plumules, and roots. The excised organs showed a half maximal GH3 protein response by 4 – 8 h and a maximum response by 24 h (Fig. 5). All organs assayed at 36 h showed greatly reduced GH3 levels. An increase in the GH3 protein could be detected in soybean suspension cells within 30 – 60 min after addition of 2,4-D. These suspension cells showed a half-maximal GH3 response by 4 h with a maximum response by 24 h (Fig. 5).

GH3 dose response

Previous experiments showed that the induction of GH3 transcription was nearly linear in response to increasing concentrations of 2,4-D [9]. To determine if the GH3 protein accumulated in a linear fashion in response to increasing 2,4-D concentrations, 4-d

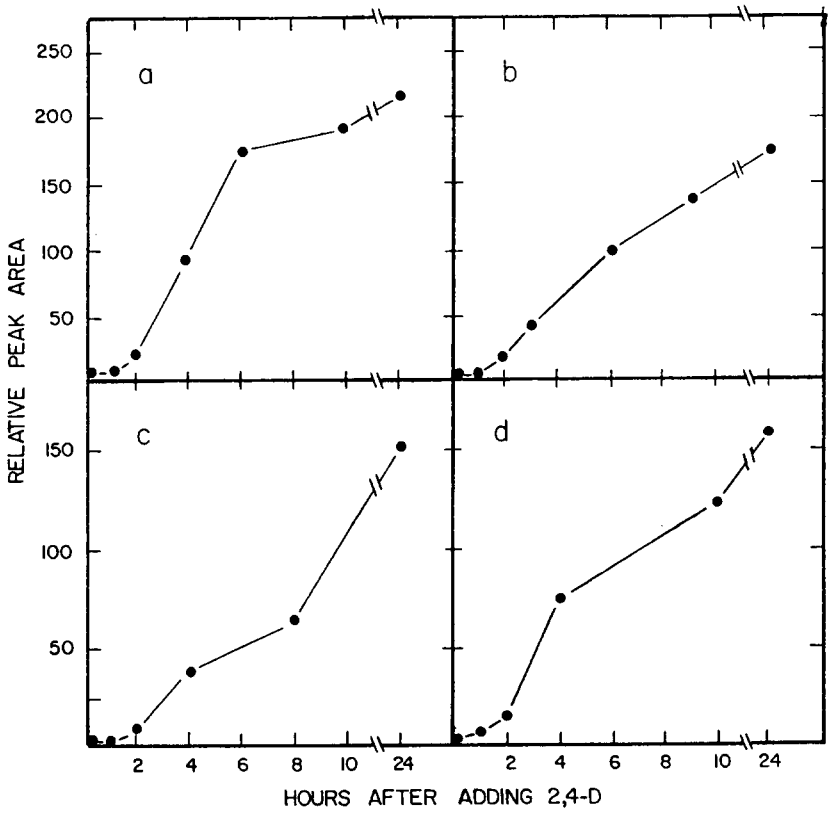

Fig. 5. Kinetics of GH3 induction by 2,4-D. 3-d elongating hypocotyls, 3-d roots or 7-d plumules were excised, preincubated 8 h in incubation media, and then 2,4-D was added to a final concentration of 100 μM. Soybean suspension cells were maintained for 3 d in MS media without 2,4-D and then 2,4-D added to a final concentration of 100 μM. Samples of all tissues were removed at time intervals, frozen and treated as in Fig. 2. Densitometry was used to quantitate the induction response. Panel a, root; b, plumule; c, hypocotyl; d, suspension cells.

root sections, 4-d elongating hypocotyl sections, and 7-d plumules were preincubated for 4 h and then incubated in varying concentrations of 2,4-D for 8 h (Fig. 6). The minimum concentration of 2,4-D capable of a measurable induction in the organs was 1 μM. In all organs assayed the GH3 protein increased nearly linearly up to the highest 2,4-D concentration tested, 1 mM.

Specificity of the GH3 induction

Previous studies have shown that GH3 transcription is specifically induced by the auxins 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), indole-3-acetic acid (IAA), and naphthaleneacetic acid (NAA) and induced much more weakly by the non-auxins, cyclohexylacetic acid, 1-cyclohexenylacetic acid and benzoic acid [9]. To determine if these compounds also induce the GH3 protein, hypocotyl sections were treated with the above compounds (100 μM) for 8 h and analyzed for GH3 induction by protein blotting (Fig. 7). The compounds, in order of strongest

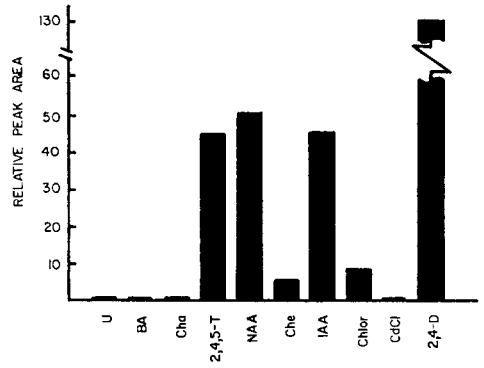

Fig. 7. Specificity of the auxin-induced GH3 response. Excised 3-d elongating hypocotyl sections were preincubated for 4 h in incubation media and then incubated for 8 h in one of the auxins or non-auxin compounds at 100 μM. The treated tissues were frozen, and treated as in Fig. 2. The responses were quantitated benzoic acid (BA), cyclohexylacetic acid (Cha), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), α-naphthaleneacetic acid (NAA), cyclohexenylacetic acid (Che), indole-3-acetic acid (IAA), σ-chlorophenoxyacetic acid (chlor), cadmium chloride (CdCl), or 2,4-D.

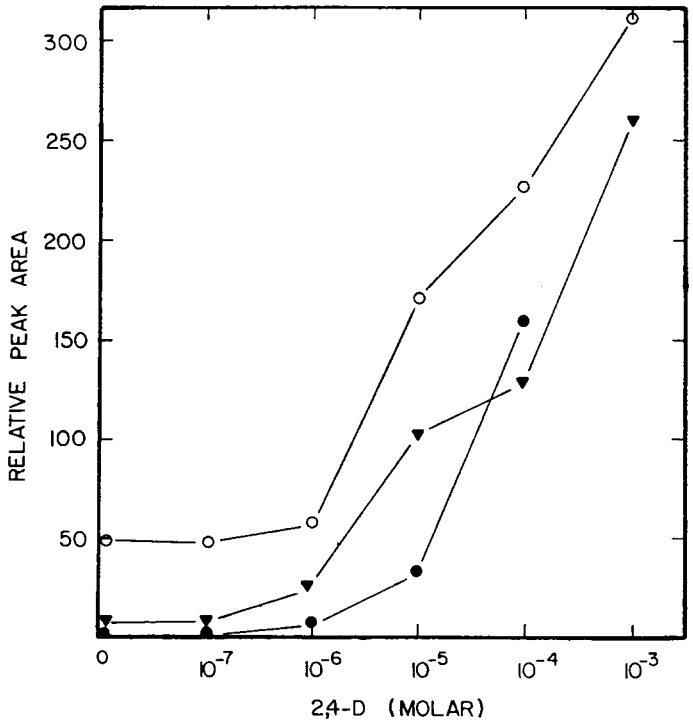

Fig. 6. Dose response curves of GH3 induction by 2,4-D. 3-d roots (open circles), 3-d elongating hypocotyls (closed circles) or 7-d plumules (closed triangles) were excised and preincubated in incubation media for 4 h and then incubated with different concentrations of 2,4-D for 8 h. Samples were then frozen, and treated as in Fig. 2. Densitometry was used to quantitate the reponses.

to weakest inducer, are 2,4-D > 2,4,5-T = IAA = NAA > σ-chlorophenoxyacetic acid > cyclohexenylacetic acid. Cyclohexylacetic acid and benzoic acid did not induce the GH3 protein. These GH3 protein induction responses are very similar to the GH3 transcription results [9]. Cadmium chloride and heat shock treatments (data not shown) did not induce GH3 protein production which agrees with previous GH3 transcription results ([9], G. Hagen, personal communication).

Other plant growth regulators such as gibberellic acid, cytokinin, ethylene, and fusicoccin were tested for their effect on GH3 protein induction in the presence or absence of 2,4-D. Previous studies had shown that neither cytokinin, which blocks auxin-induced cell elongation in hypocotyls [20], nor ethylene affected GH3 transcription [9]. These same hormones did not inhibit or stimulate the induction of the GH3 protein (data not shown). Fusicoccin [14] and gibberellic acid [10], which cause cell elongation in hypocotyl sections, did not affect the GH3 protein induction response when applied alone or with 2,4-D.

GH3 species specificity

The GH3-specific antiserum was used to determine if the GH3 protein was ubiquitous among plants (Fig. 8). All dicots assayed showed a 2,4-D-inducible 70-kd protein that reacted with the GH3 antisera. In some cases a second auxin-inducible protein at about 68 kd (petunia) or 72 kd (mung bean) is detected. Thus, it is possible that more than one auxin-inducible polypeptide may share homology with GH3 in different plant species. The GH3 antisera also reacted with a 70-kd protein from wheat and maize. However, this protein was not highly inducible upon 2,4-D treatment, and may or may not be analogous to the protein detected in dicots. The significance of other non-auxin-inducible bands detected with anti-GH3 is unclear (see section Intracellular location and organ specificity).

Discussion

In this study, polyclonal antisera were raised to a polypeptide coded by the auxin-inducible cDNA,

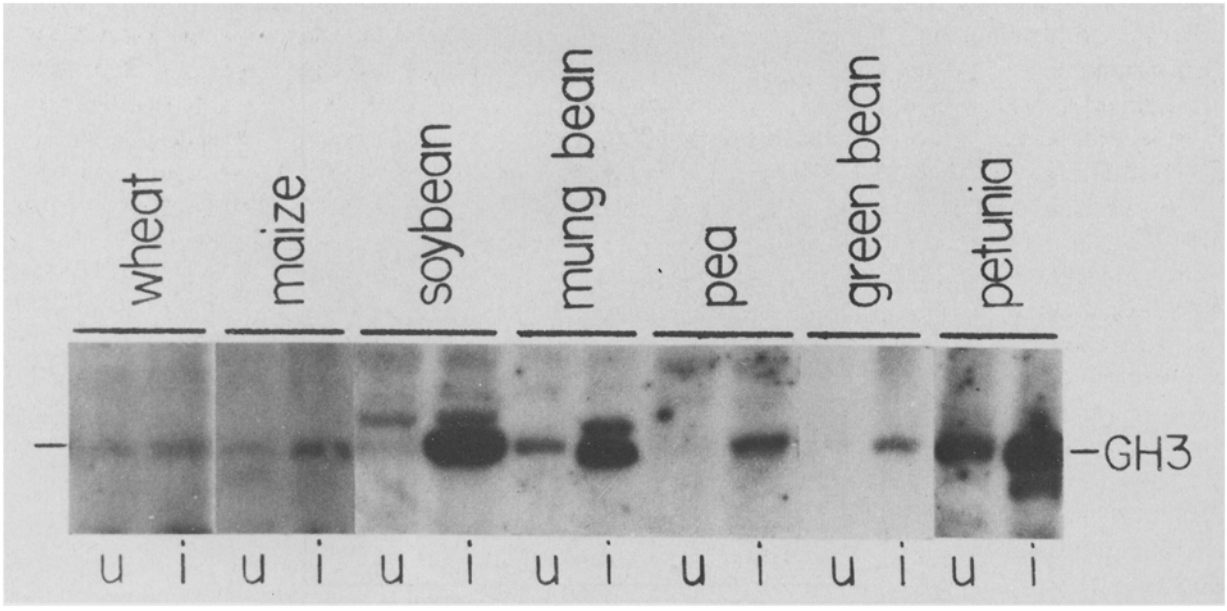

Fig. 8. Species specificity of the GH3 protein. 4-d roots from pea and green bean and 4-d elongating hypocotyl soybean sections were incubated for 8 h in incubation media alone (u) or incubation media containing 100 μM 2,4-D (i). Intact 5-d wheat, maize, and mung bean seedlings and 60-d petunia plants were sprayed with 10 mM potassium phosphate (pH 6) (u) or sprayed with potassium phosphate containing 1 mM 2,4-D (i), and root tissue was harvested 12 h later. Organ samples were frozen and treated as in Fig. 2.

pGH3. These antisera react with a protein of 70 kd that is induced 30–50-fold by addition of 2,4-D to soybean seedlings. RNA blot analysis has shown the GH3 mRNA is large enough to code for a protein of 70 kd. The GH3 protein is not associated with the cell wall, nucleus, or other pelletable fractions, and its location does not change in response to auxin treatment as has been seen with heat shock proteins in response to heat shock [21, 22]. Studies on the intact seedlings at 3 d–14 d of development showed that the roots contained the highest and the hooks, epicotyls, and plumules the lowest basal levels of the GH3 protein. The GH3 protein increases throughout the seedling with age, and organs from 14-d plants contained higher GH3 levels than the same organs in 3–7-d plants. It is interesting that in all organs except the root, the GH3 protein is lowest in rapidly dividing meristematic tissues which presumably contain the highest levels of IAA. Tissue from rapidly dividing root tips or quiescent mature root sections contained nearly equal amounts of the GH3 protein. Results showing that the basal level of the GH3 protein is highest in roots suggest GH3 might perform a particular function in the root. However, by 14 d the GH3 level in the epicotyl approaches that found in the root. The root is thought to be more sensitive to exogenous auxin than other plant organs; however, elongating hypocotyl sections, plumules, and roots all showed similar dose response and induction kinetics. The dose respons and induction kinetics for the GH3 mRNA [8, 9] and GH3 protein are consistent. However, due to the difference in sensitivity between the assays for GH3 mRNA production and GH3 protein production, the GH3 mRNA is detected more rapidly and at lower concentrations of 2,4-D [8, 9]. Likewise, transcription rate increases on the GH3 gene(s) following auxin addition are much more rapid than either mRNA or protein accumulation [8, 9]. Soybean suspension cells did induce more rapidly (30–60 min) than any other seedling organs probably because the cells capable of producing the GH3 protein were more accessible to the 2,4-D.

Previous studies had shown that the GH3 transcription was induced by several auxins and non-auxin analogs of synthetic auxins [9]. These same compounds were assayed for their ability to induce the GH3 protein and the results are consistent with the GH3 transcription induction study. Previous experiments showed that the non-auxins cyclohexylacetic acid and benzoic acid did weakly induce low levels of GH3 transcription [9]; however, the GH3 protein assay is probably not sensitive enough to pick up this slight induction.

The growth regulators cytokinin and ethylene, which inhibit auxin-induced cell elongation in hypocotyls, did not affect GH3 protein induction. Gibberellic acid and fusicoccin, which cause cell elongation in hypocotyl sections, had no effect on GH3 protein induction either. These results suggest that auxin-induced cell elongation occurs by a different mechanism than that of fusicoccin or gibberellic acid or that the GH3 protein does not have a function in auxin-induced cell elongation. The organ distribution of the GH3 protein also argues against a specific role for GH3 protein in cell elongation processes.

A 70-kd protein was detected in all dicots and monocots tested using the GH3 antisera. However, wheat and maize did not show a dramatic increase in the level of the 70-kd protein after incubation with 2,4-D. It is unclear whether all these proteins are highly homologous or have retained specific homologous antigenic determinants. The auxin inducibility in a number of dicots tested does, however, suggest that the GH3 protein may play a similar role in response to auxin for a wide variety of plants.

Using the GH3 specific antisera we are currently purifying the GH3 protein, and we hope to determine its role in the auxin response.

Acknowledgement

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