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Hormonal regulation of β1,3-glucanase messenger RNA levels in cultured tobacco tissues

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We describe the isolation of a cDNA clone of β1,3-glucanase mRNA from *Nicotiana tabacum* L. cv. 'Havana 425' and its use to measure the kinetics of mRNA accumulation in cultured tobacco tissues treated with the plant hormones auxin and cytokinin. Northern blot analysis showed that the tissues contain a single ~1.6 kb-sized β1,3-glucanase mRNA. The levels of β1,3-glucanase and β1,3-glucanase mRNA increase by up to seven- and 20-fold, respectively, over a 7-day period in tissues subcultured on hormone-free medium and medium containing auxin or cytokinin added separately. Over the same interval of time, the content of both the enzyme and its mRNA remains at a constant low level in tissues subcultured on medium containing both auxin and cytokinin. The results show that auxin and cytokinin block β1,3-glucanase production at the level of the mRNA.

Key words: auxin/cytokinin/β1,3-glucanase/cDNA clone/*Nicotiana tabacum* (tissue culture)

Introduction

The hormones auxin and cytokinin are thought to have a central role in regulating plant growth and development (Letham *et al.*, 1978). Although there are reports that these hormones can alter gene expression at the mRNA level (Meyer *et al.*, 1984; Hagen *et al.*, 1984; reviewed by Theologis and Ray, 1982), with very few exceptions (Verma *et al.*, 1975; Parthier *et al.*, 1982) the gene products have only been identified as spots or bands on polyacrylamide gel electrophoretograms (PAGE).

Earlier we showed that auxin and cytokinin regulate the accumulation of β1,3-glucanase (E.C. 3.2.1.39) in cultured tobacco tissues (Eichholz *et al.*, 1983; Felix, 1985; Felix and Meins, 1985). When pith tissues cultured on a complete medium containing auxin and cytokinin are subcultured on hormone-free medium or on medium containing either auxin or cytokinin, the tissues rapidly accumulate up to about 10% of their soluble protein as β1,3-glucanase over a seven-day period. In contrast, when these tissues are subcultured on medium containing both auxin and cytokinin, the β1,3-glucanase content remains at a constant, low level. We also showed that the incorporation of [^{35}S]methionine into β1,3-glucanase by cultured tissues was blocked by auxin and cytokinin treatment suggesting that these hormones inhibit synthesis of the enzyme (Felix, 1985).

These observations are of interest for two reasons. First, they provide one of the few experimental systems for studying the action of auxin and cytokinin on the production of a well-characterized enzyme. Second, β1,3-glucanase activity is induced when plants are infected with pathogens and the enzyme has been implicated in the defence reaction of the host plant (Bell, 1981; Sequeira, 1983).

In this report, we describe the isolation of a cDNA clone for tobacco β1,3-glucanase mRNA and the use of this clone to measure mRNA accumulation in tissues incubated on media containing different combinations of auxin and cytokinin. Our results indicate that these hormones inhibit β1,3-glucanase production at the level of mRNA.

Results

Construction and isolation of cDNA clones for glucanase

A cDNA library was prepared using RNA isolated from tobacco tissues induced to produce β1,3-glucanase by incubation on basal medium without added hormones. Double stranded cDNA produced from size-fractionated poly(A)$^+$ RNA was inserted into the *Pst*I site of pBR322 by a homopolymeric dC-dG tailing method (Maniatis *et al.*, 1982) and transformed into *Escherichia coli* host strain DH1. The cDNA library was first screened by differential colony hybridization. As a source of the RNAs used to produce radioactive probes, we chose tissues cultured for seven days on basal medium and on auxin + cytokinin medium. Under these conditions the induced tissues on basal medium contained ~10-fold more glucanase than the non-induced tissues (Felix, 1985). We verified that the RNA preparations differed in glucanase mRNA content by *in vitro* translation of the poly(A)$^+$ RNA fractions. SDS-PAGEs of the ^{35}S-labeled translation products are shown in Figure 1. A band of 37 kd, corresponding to the precursor form of tobacco β1,3-glucanase (Shinshi and Katō, 1983a), was present in the translation products obtained with induced RNA but absent in the products obtained with non-induced RNA. This 37 kd translation product was immunoabsorbed by anti-glucanase IgG, which was shown to be specific for β1,3-glucanase in protein extracts of tobacco (Felix and Meins, 1985). No labeled material was immunoabsorbed by pre-immune serum.

Ninety of 2912 cDNA clones screened by differential colony hybridization gave a stronger hybridization signal with radioactively labeled cDNA of induced RNA than with radioactively labeled cDNA of non-induced RNA. These were screened by hybrid-select translation, and one clone pGL43, which contained an ~1 kb insert, was judged to be a cDNA clone of glucanase mRNA by the following criteria: (i) pGL43 hybridized with an RNA which gave as a major *in vitro* translation product a protein with the size of the 37 kd glucanase precursor (Figure 2). This product was specifically immuno-absorbed by anti-glucanase IgG. (ii) Northern blot analysis showed that pGL43 hybridizes with a single ~1.6-1.7 kb RNA species present in the total and poly(A)$^+$ RNA (Figure 3). This species was present at higher concentrations in RNA from induced tissues than in RNA from non-induced tissues. Moreover, these concentrations were correlated with the amount of translatable glucanase mRNA in tissues incubated under the same conditions (see Figure 1). Further characterization of the clone and sequence data will be published elsewhere (Shinshi *et al.*, in preparation).

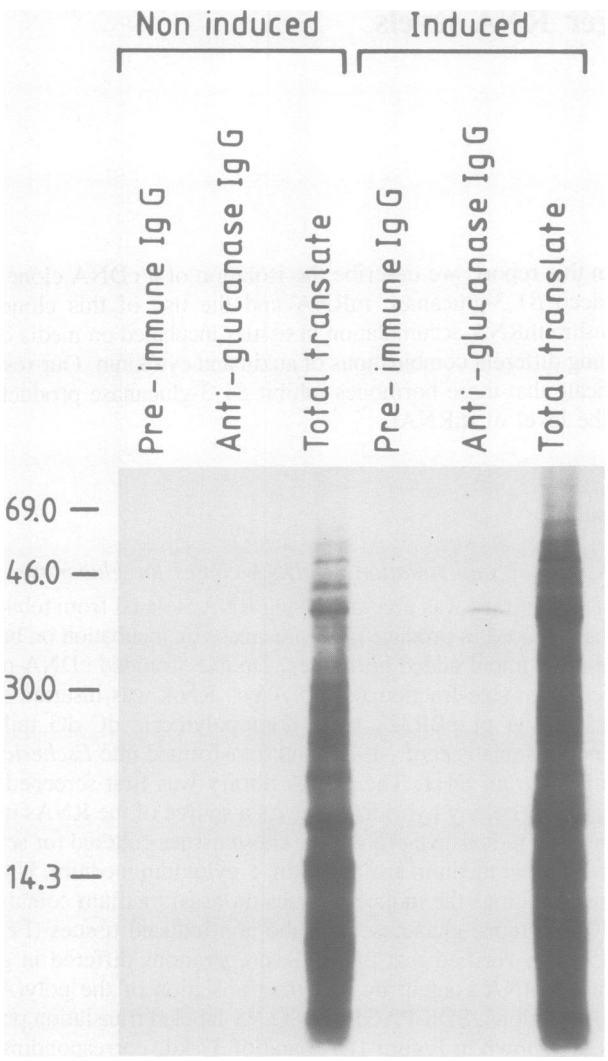

Fig. 1. SDS-PAGE of *in vitro* translation products obtained with 0.8 μg of poly(A)$^+$ RNA from non-induced (7 days, auxin + cytokinin medium) and induced (7 days, basal medium) tissues. Equal amounts (~2.5 x 10^5 c.p.m.) of ^{35}S-labeled protein were used for immuno-precipitation with pre-immune IgG or anti-glucanase IgG and were applied to the gels. Scale at left: mol. wt. (in kd) of protein standards.

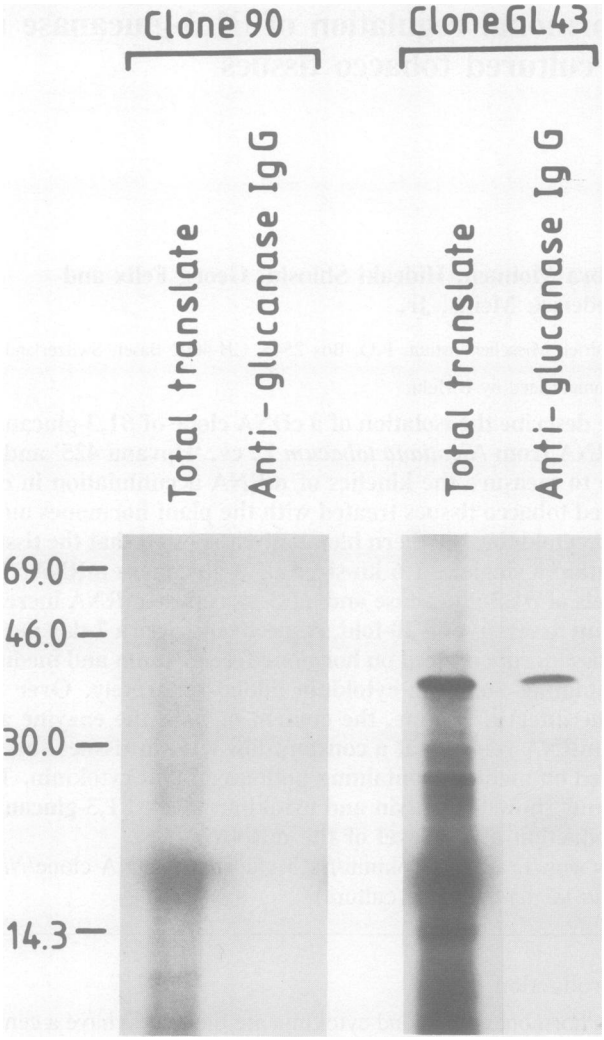

Fig. 2. SDS-PAGE of *in vitro* translation products obtained with hybrid-selected RNA. Plasmid DNA from control (90) and glucanase (pGL43) clones was hybridized with total RNA from tissues incubated seven days on basal medium. Aliquots of the *in vitro* translation mixture and anti-glucanase immunoprecipitation were applied to the gels as indicated. Scale at left: mol. wt. (in kd) of protein standards.

Effect of hormones on β1,3-glucanase mRNA accumulation

We compared the effect of hormones on β1,3-glucanase production and on the level of β1,3-glucanase mRNA in tobacco tissues. After subculturing for 3 days on media containing different combinations of the hormones, tissues were either used to prepare total RNA or incubated for an additional 18 h with [^{35}S]methionine. Protein extracts were prepared from the labeled tissues, the extracts immuno-absorbed with anti-tobacco glucanase IgG, and the absorbed material analyzed by SDS-PAGE.

The immuno-absorbed, ^{35}S-labeled proteins migrated as a single band at the 33 kd position in the gel corresponding to the position of purified, mature tobacco β1,3-glucanase (Shinshi and Katō, 1983b; Felix and Meins, 1985) (Figure 4A). The intensity of labeling depended on the hormonal constitution of the medium. The strongest labeling was obtained with tissues incubated without added hormones. Although auxin and cytokinin added separately reduced the labeling somewhat, marked inhibition was only obtained when both hormones were included in the medium. Essentially the same pattern of hormone action was obtained for β1,3-glucanase mRNA as judged by Northern analysis of total RNA using pGL43 as probe (Figure 4B). The amount of 1.6 kb glucanase mRNA was high in tissues incubated without hormones, somewhat reduced in tissues incubated with the hormones added separately, and very low in tissues incubated with the mixture of both hormones.

The kinetics of glucanase and glucanase mRNA accumulation was compared in tissues incubated for 0–7 days on the various test media. The amount of glucanase was measured by rocket immunoelectrophoresis. Figure 5A shows the accumulation of glucanase expressed as μg enzyme/mg of soluble protein. Comparable results were obtained when the data were expressed on a fresh-weight basis. The most rapid accumulation was obtained with basal medium which gave a 7-fold increase by day 7. Accumulation on media containing either auxin or cytokinin alone was somewhat slower, but the same high levels of glucanase were obtained by day 7. In contrast, the glucanase content of tissues incubated on media with both hormones remained at a constant low level.

The content of glucanase mRNA was measured by dot-blot hybridization using pGL43 DNA as a probe. The results express-

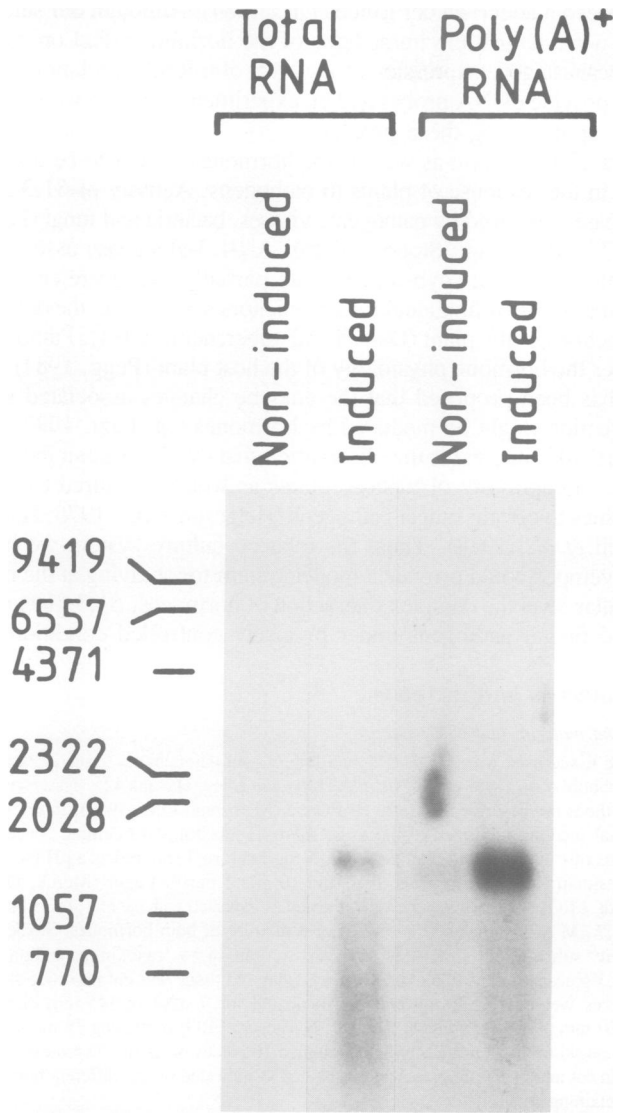

Fig. 3. Northern analysis of 6.4 μg total RNA and 0.8 μg poly(A)$^+$ RNA with the glucanase cDNA probe. RNA was prepared from non-induced (7 days, auxin + cytokinin medium) and induced (7 days, basal medium) tissues. Scale at left: sizes (in bases) of denatured DNA standards.

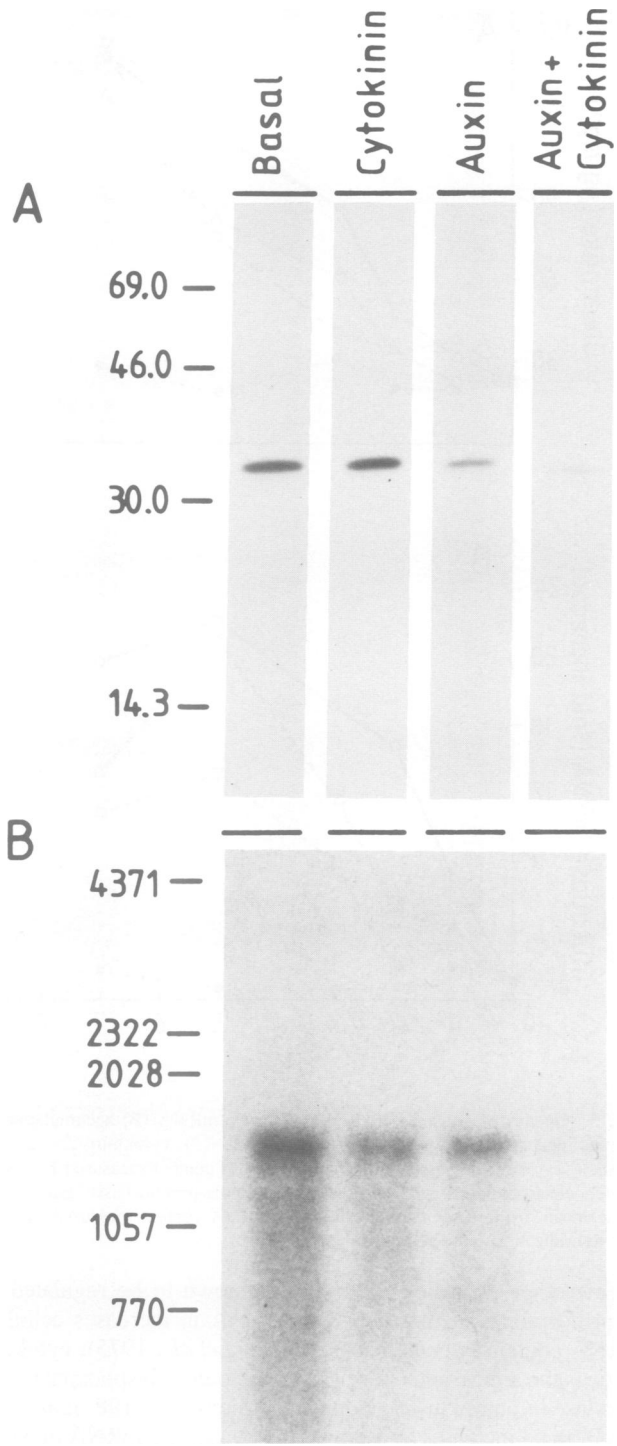

Fig. 4. Effect of hormone treatment on *in vivo* incorporation of [^{35}S]methionine into glucanase and glucanase mRNA content. Tissues were incubated 3 days on basal-, auxin-, cytokinin- and auxin + cytokinin medium. **(A)** Autoradiograms of SDS-PAGEs obtained with anti-glucanase immunoprecipitates of equal amounts (~2.0 x 10^5 c.p.m.) of ^{25}S-labeled proteins. Scale at left: mol. wt. (in kd) of protein standards. **(B)** Northern blot analyses of total RNA with pGL43 as probe. Equal amounts (6.4 μg) of total RNA were applied to each lane. Scale at left: sizes (in bases) of denatured DNA standards.

ed as fold increase in mRNA content relative to time zero are shown in Figure 5B. The glucanase mRNA levels in tissues incubated on medium without hormones increased rapidly by ~20-fold at day 5 and then declined slightly. There were also somewhat lower increases in the mRNA content of tissues incubated on media containing either hormone added separately. In striking contrast, the glucanase mRNA content of tissues incubated on medium containing both hormones remained roughly constant at the low, zero-time level. Thus, glucanase and glucanase mRNA exhibited similar patterns of accumulation in response to hormone treatment.

Discussion

There are reports that the hormones auxin, cytokinin, gibberellic acid, abscisic acid and ethylene alter the activity of β1,3-glucanase in whole plants and detached plant parts (Abeles *et al.*, 1970; Moore and Stone, 1972a; Davies and MacLachlan, 1968; Jones, 1971; Boller *et al.*, 1983). Our earlier studies showed that auxin and cytokinin inhibit the appearance of β1,3-glucanase activity in cultured tobacco tissues by blocking production of the enzyme (Eichholz *et al.*, 1983; Felix, 1985; Felix and Meins, 1985). The present report shows that the effects of these hormones on glucanase production and the accumulation of glucanase mRNA are closely correlated. This provides strong evidence that auxin and cytokinin inhibit glucanase production at the level of mRNA.

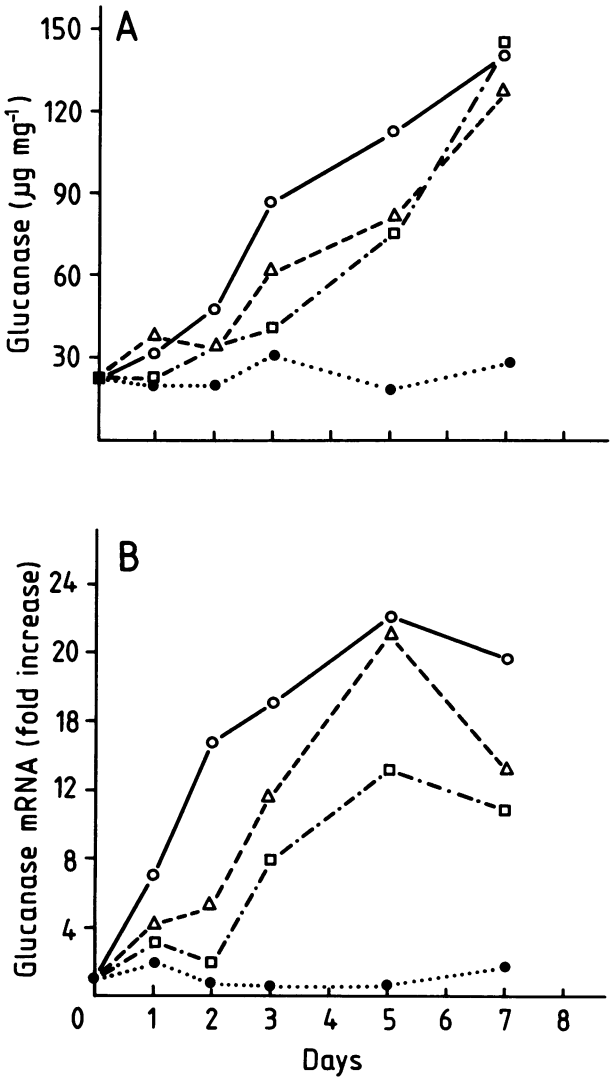

Fig. 5. Kinetics of glucanase **(A)** and glucanase mRNA **(B)** accumulation. Tissues incubated on basal (○−○), auxin (□···□), cytokinin (△--△), and auxin + cytokinin (●···●) medium. Glucanase content measured by rocket immunoelectrophoresis and expressed on a soluble-protein basis. Each point is the mean of three replicates. Glucanase mRNA content measured by dot blot hybridization with pGL43 as probe.

Few other identified proteins are known to be regulated by plant hormones at the mRNA level. Auxin increases cellulase mRNA content in pea epicotyls (Verma *et al.*, 1975); cytokinin induces the appearance of mRNA for ribulose bisphosphate carboxylase in pumpkin cotyledons (Parthier *et al.*, 1982); and gibberellic acid increases transcription of α-amylase mRNA in barley aleurone layers which is inhibited by abscisic acid (Baulcomb and Buffard, 1983; Chandler *et al.*, 1984). Abscisic acid has also been shown to induce and/or maintain the level of mRNA for storage proteins in cultured rape embryos (Crouch *et al.*, 1983). The regulation of β1,3-glucanase we describe is unique in that the combined effect of auxin and cytokinin is required for inhibition of mRNA accumulation.

The abundance of mRNA could be regulated at several levels including transcription, RNA processing, transport and mRNA stability (Darnell, 1982). Studies of run-off transcription with isolated nuclei show that ethylene and gibberellic acid can induce transcription of specific genes (Nichols and Laties, 1984; Zwar and Hooley, 1984). There is also evidence that an auxin receptor-auxin complex can promote transcription in isolated tobacco nuclei (van der Linde *et al.*, 1984). Although our studies do not establish the immediacy of the hormone effect on β1,3-glucanase gene expression or the level of mRNA regulation, they do provide cDNA probes and an experimental system well suited for approaching these problems.

β1,3-Glucanase as well as the hormones appear to be involved in the response of plants to pathogens. Activity of β1,3-glucanase is induced by pathogenic viruses, bacteria and fungi (Pegg, 1977; Moore and Stone, 1972b). A β1,3-glucanase associated with the wall of soybean cells can partially hydrolyze cell-wall components of fungi and release elicitors that activate the defence reactions of the plant (Darvill and Albersheim, 1984). Pathogens alter the hormone physiology of the host plant (Pegg, 1981) and it has been proposed that the enzyme changes associated with infection might be mediated by hormones (Mishagi, 1982). Of particular interest is the observation that cytokinins can increase the susceptibility of tobacco plants as well as cultured tobacco tissues to certain fungal pathogens (Helgeson *et al.*, 1976; Haberlach *et al.*, 1978). Thus, the tobacco culture system we have developed could provide a model system for studying at the molecular level the complex interaction of hormones, β1,3-glucanase and fungal pathogens under precisely controlled conditions.

Materials and methods

Plant material and tissue culture

The tissue used was 275N, a cloned line of pith parenchyma tissue isolated by Eichholz *et al.* (1983) from *Nicotiana tabacum* L. cv. Havana 425. Tissue culture methods are described in detail elsewhere (Meins and Lutz, 1980). In brief, the basal medium consisted of the salts, sucrose, inositol and thiamine concentrations of Linsmaier and Skoog (1965), 5 mg/l chlorophenol red as a pH indicator (Eastman Kodak, Rochester, NY) and 10 g/l of purified agar (Merck, Darmstadt, FRG). The media were supplemented as indicated with either 1.4 μM kinetin, 10.7 μM α-naphthaleneacetic acid, or a mixture of both hormones. Stock lines were subcultured at 21-day intervals on auxin + cytokinin medium. In β1,3-glucanase induction experiments, 21-day-old tissue was cut into cube-shaped pieces weighing ~30 mg and pre-incubated for 7 days in 145 mm diameter x 20 mm plastic Petri dishes (Greiner, Nurtingen, FRG) containing 75 ml of auxin + cytokinin medium. Each dish contained 16 pieces of tissue. The tissues were then cut into ~30 mg pieces and incubated as indicated on the different hormone-containing media.

RNA isolation

RNA was isolated from tissues frozen in liquid N_2 and stored at −80°C by the method of Short and Torrey (1972) with the following modifications: (i) after the SDS-phenol extraction two additional extractions were made with phenol: chloroform:isoamyl alcohol (25:24:1) and the RNA precipitated with ethanol. (ii) The precipitate was washed consecutively at 4°C with 5 mM EDTA, 3 M sodium acetate; 5 mM EDTA, 2 M LiCl; and 0.2 M NaCl, 70% (v/v) ethanol. Polyadenylated RNA [poly(A)$^+$ RNA] was isolated by affinity chromatography on oligo-dT-cellulose type 3 (Collaborative Research, Lexington, MA) (Aviv and Leder, 1972). RNA concentration was estimated by absorption at 260 nm (Maniatis *et al.*, 1982). The yield of total RNA was ~190 μg/g F.W. tissue of which ~0.9% was recovered as poly(A)$^+$ RNA.

Protein extraction and analysis of protein

Protein extracts were prepared and analyzed by SDS-polyacrylamide gel electrophoresis essentially as described by Eichholz *et al.* (1983). β1,3-Glucanase was assayed by 'rocket' immunoelectrophoresis using purified enzyme as standard as described by Felix and Meins (1985). Three replicate samples of tissue were assayed. A modified Werner and Machleidt (1978) method was used for immunoabsorption with protein A-Sepharose. An ~30 μl-suspension of protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was washed twice in binding buffer containing 0.15 M NaCl, 0.1% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 5 mg/ml bovine serum albumin (Fraction V, Sigma, St. Louis, MO), 10 mM Tris-HCl (pH 8.0) by centrifugation and resuspension and then incubated with shaking (Vortex-Genie, Bender and Hobein, Zürich, Switzerland) with ~100 μg rabbit pre-immune IgG or rabbit anti-β1,3-glucanase IgG (Felix and Meins, 1985) in 1 ml of binding buffer for 45−60 min at 4°C. After washing the protein A-IgG complex twice with 1 ml binding buffer without BSA and twice with 1 ml binding buffer, it was incubated for 60 min at 4°C with the protein samples in 1 ml of binding buffer. The resultant complexes were washed twice with binding buffer and twice with 0.15 M NaCl, 10 mM Tris-HCl (pH 8.0),

and then eluted by incubating for at least 2 h at 25°C in 75 μl of 1.5-strength gel sample buffer with occasional mixing. The extract was incubated for 2 min in a boiling water bath before applying to polyacrylamide gels.

In vitro translation

Poly(A)$^+$ RNA (0.8 μg) was translated in a wheatgerm-extract system (Bethesda Research Laboratories, Bethesda, MD) as recommended by the manufacturer and ~10 μCi L-[^{35}S]methionine (sp. act. ~1275 Ci/nmol; Amersham, Buckinghamshire, UK) was added per 30 μl reaction. The incorporation of [^{35}S]methionine was monitored (Bollum, 1959) and when the reaction was complete the mixture of protease inhibitors recommended by Riezman *et al.* (1983a) was added. The reaction mixture was either used directly in immuno-absorption experiments or analyzed by gel electrophoresis after clarifying by centrifugation for 5 min in an Eppendorf microfuge.

Construction and selection of a cDNA clone for β1,3-glucanase

Poly(A)$^+$ RNA isolated from tobacco tissue incubated for seven days on basal medium, was fractionated by preparative ultracentrifugation for 17 h at 57 000 *g* on a 5–25% (w/v) linear sucrose gradient containing 1 mM EDTA, 10 mM Tris-HCl (pH 7.5). Fractions containing β1,3-glucanase message as judged by *in vitro* translation and immuno-absorption with anti-β1,3-glucanase antibody, were pooled. Double stranded cDNA synthesis, cloning into plasmid pBR322, differential colony hybridization and plasmid isolation were essentially as described by Maniatis *et al.* (1982). In brief, 3 μg of poly(A)$^+$ RNA enriched for β1,3-glucanase mRNA was used to produce 0.8 μg of double-stranded cDNA using reverse transcriptase (Life Sciences, St. Petersburg, FL) and DNA polymerase I (New England Biolabs, Beverly, MA) for first- and second-strand synthesis, respectively. The cDNAs were inserted into the *Pst*I site of pBR322 by the homopolymeric dC-dG tailing method, and the recombinant plasmids used to transform the *E. coli* strain DH1. The cDNA library was first screened by differential colony hybridization using radioactive cDNA produced from poly(A)$^+$ RNA from tissues induced to produce β1,3-glucanase (seven-day incubation on basal medium) and from non-induced tissues (seven-day incubation on auxin + cytokinin medium). Promising clones were then screened by the hybrid-select-translation method of Cochet *et al.* (1979) as modified by Riezman *et al.* (1983b) except that plasmid DNA was denatured by boiling in 0.3 M NaOH, 3 M NaCl for 1 min. Hybridization was carried out on 9 x 9 mm square BA/85 nitrocellulose filters (Schleicher and Schull, Dassel, FRG) with 200–250 μg of total RNA per filter. Hybridized RNA on the filters was eluted, ethanol precipitated, dissolved in 10 μl of water and analyzed by *in vitro* translation.

Northern and dot blot analysis

The method for Northern and dot blot analysis was that of Thomas (1983). For Northern analysis, 0.8 μg of poly(A)$^+$ RNA or 6.4 μg of total RNA denatured in 1 M glyoxal, 50% (v/v) dimethyl sulphoxide, 0.01 M Na HPO_4 pH 7.0 was used per lane in a 1.1% (w/v) agarose gel. In dot blot analysis experiments 4.0 μg of total RNA per sample was used. Ethidium bromide staining was used to confirm that equal amounts of RNA were used in each analysis. The nick-translated (Maniatis *et al.* (1982), *Pst*I insert of plasmid pGL43 was used for hybridization. Autoradiograms were scanned with a densitometer (TLC-Scanner, CAMAG, Muttenz, Switzerland). Glucanase mRNA content, estimated from the peak heights of scans of dots by comparison with values obtained with known amounts of RNA, is expressed as fold increase above the time zero value for three replicate dots on the same filter.

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