Plant Physiol. (1993) 102: 513–520

Further Characterization of Expression of Auxin-Induced Genes in Tobacco (*Nicotiana tabacum*) Cell-Suspension Cultures[1]

Kees J. M. Boot*, Bert J. van der Zaal, Joyce Velterop, Ab Quint, Albert M. Mennes, Paul J. J. Hooykaas, and Kees R. Libbenga

Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

We have described the modulation of four auxin-regulated genes during the growth cycle of suspension-cultured tobacco (*Nicotiana tabacum* [L.] var White Burley) cells. The genes were transiently expressed 2 to 8 h after transfer of stationary phase cells to fresh medium, during the transition from the quiescent phase of cells leaving the mitotic cycle to the synthesis phase of the cell cycle. After this transient induction, the cells showed a decreased sensitivity to auxin. Although the expression pattern suggests that induction of these genes might be important for cell division, overproduction of antisense mRNA for one of these genes (pCNT103) did not influence cell division in transgenic tobacco cells. Furthermore, stimuli such as salicylic acid were capable of inducing gene expression but were unable to restore cell division. Although these data do not conclusively exclude a role for these genes in cell division, their significance in this process is discussed in view of their homology with other auxin-induced genes and in view of the specificity of hormone-induced early responses.

Plant hormones are known to have pleiotropic effects on growth and development. For example, the naturally occurring auxin, IAA, influences cell division, cell elongation, and differentiation. A variety of early events (time scale: seconds to hours) preceding the biological response to auxin has been described (Brummell and Hall, 1987). However, the specificity of these early responses and their significance for the occurrence of the biological response are in general far from clear. For instance, the stimulation of proton extrusion and the hyperpolarization of the plasma membrane potential are well-described rapid auxin effects. Recently, however, it was shown that both growth-promoting auxins and growth-inactive structural analogs were capable of inducing these processes (Felle et al., 1991; Peters and Felle, 1991), demonstrating that stimulation of the proton pump is a rather unspecific effect of auxin, although the activation of the H^+-ATPase is indispensable for the occurrence of cell elongation.

During the past few years, a number of research groups, including our own, have studied early effects of auxins on the induction of specific proteins and mRNAs (for recent reviews, see Guilfoyle, 1986; Theologis, 1986; Key, 1989). Auxin-responsive genes that respond within minutes after hormone treatment have been cloned and characterized (Hagen et al., 1984; Theologis et al., 1985; McClure and Guilfoyle, 1987; Van Der Zaal et al., 1987a; Ainley et al., 1988; Takahashi et al., 1989).

In our laboratory, we found that when adding auxins to auxin-starved cells in cell cultures from tobacco (*Nicotiana tabacum*), a number of mRNAs are rapidly induced before renewed cell division activity. For one of these genes, pCNT103, a high correlation was observed between the dose-dependent auxin-specific mRNA accumulation and cell division (Van Der Zaal et al., 1987a).

In this paper, we report a detailed study of the modulation of some of these genes (i.e. pCNT103, -107, -114, and -115) during the growth cycle of an initially synchronized cell culture (Van Der Zaal et al., 1987b). We show that the genes are transiently induced in an auxin-dependent manner during the transition of cells from G_0 to S. Although this suggests a role for these genes in the auxin-signal transduction pathway leading to cell division, we found that using antisense technology to suppress pCNT103 mRNA apparently has no effect on auxin-induced cell division. In addition, we found that nonmitogenic stimuli such as SA are able to induce pCNT103 and related mRNAs. These results are discussed in relation to the specificity and significance of early auxin-induced genes, with particular emphasis on cell division.

MATERIALS AND METHODS

Cell-Suspension Cultures

Nicotiana tabacum [L.] (var White Burley) cell-suspension cultures were grown on Linsmaier-Skoog (Linsmaier and Skoog, 1965) medium containing 0.22 μM 2,4-D. To maintain the cultures, early-stationary phase cells were transferred after 7 d to fresh medium with the same 2,4-D concentration (LT 0.05 cell lines, maintenance culture). The cells were auxin starved by growing them on hormone-free medium (0.0 cells) for 5 d, as described by Van Der Zaal et al. (1987b).

[1] This work was supported by the European Economic Communities Biotechnology programs BAP and Bridge.

* Corresponding author; fax 31–71–274999.

Abbreviations: FC, fusicoccin; G_0, quiescent phase of cells leaving the mitotic cycle; GST, glutathione S-transferase; GUS, β-D-glucuronidase; PR, pathogenesis related; S, synthesis phase of cell cycle; SA, salicylic acid.

For short-term experiments (24 h or less), cells were grown in 250-mL Erlenmeyer flasks containing 50 mL of medium at 25°C in the dark on a gyratory shaker (120 rpm, 5 cm displacement). For long-term experiments (7–8 d), cells were grown in a 3-L turbine-stirred round-bottom fermenter (Applicon) containing 2.5 L of medium. The temperature was maintained at 25°C, and the cells were grown in the dark. The rotation speed of the two six-blade turbine impellers was set at 75 rpm. The culture was aerated at a rate of 0.35 volume (air) volume (suspension)$^{-1}$ m^{-1}. Occasionally, 0.25 mL of silicone anti-foam agent (BDH, Poole, UK) was added. For the in vitro transcription run-on experiments, tobacco var Bright Yellow 2 cell suspension was used. This cell line was transformed by the co-cultivation method with a construct pMPHH 10MK, which contained the GUS gene under control of the TR1′ promoter and the TR0′-TR1′ terminator sequence (Van Der Zaal et al., 1991). This construct was used as an internal control. The cells were grown on Linsmaier-Skoog medium supplemented with 180 mg L^{-1} of KH_2PO_4, 0.6 mg L^{-1} of aneurine-HCl, 0.05 mg L^{-1} of 2,4-D, and 50 μg mL^{-1} of hygromycin at 25°C in the dark. The expression patterns of the mRNAs in this cell suspension were the same as in the White Burley maintenance culture cell lines (data not shown).

The sense and antisense cell lines were derived from White Burley tobacco plants transformed with constructs containing the cauliflower mosaic virus 35S promoter, the *nos* terminator, and the pCNT103 coding region in two orientations (Van Der Zaal, 1989). A number of calli were obtained from leaves of several independent transgenic plants, and cell-suspension cultures were derived from these calli and cultured on Linsmaier-Skoog medium containing 2.2 μM 2,4-D.

RNA Isolation and Northern Blot Analysis

After the cells were harvested and washed, they were ground under liquid nitrogen and the RNA was isolated by phenol extraction and LiCl precipitation (Van Slogteren et al., 1983). The RNA was electrophoresed in 2% agarose/6% formaldehyde gels (Maniatis et al., 1982) and blotted overnight on GeneScreen membranes (NEN/DuPont, 's Hertogenbosch, The Netherlands). All hybridization and washing conditions were as described by Van Der Zaal et al. (1987a). All probes were labeled by nick translation of cDNA clones with [^{32}P]dCTP (Amersham, 's Hertogenbosch, The Netherlands) (Van Der Zaal et al., 1987a).

Southern Blot Analysis

*Eco*RI digests of the cDNAs pCNT103, -107, -114, -115, and pMPHH 10MK were electrophoresed on 2% agarose gels and transferred to GeneScreen by capillary blotting according to the procedure of Maniatis et al. (1982).

Isolation of Nuclei and in Vitro Transcription

Nuclei were isolated as described previously (Mennes et al., 1978) with the modifications described by Van Der Zaal et al. (1987a). For the run-on experiments, 2 × 10^6 nuclei were incubated for 2 h at 26°C as described by Van Der Zaal et al. (1987a). Labeled transcripts were isolated according to the method of Hagen and Guilfoyle (1985), and similar amounts of labeled transcripts were hybridized to Southern blots as described by Van Der Zaal et al. (1987a).

Induction Experiments

All induction experiments were performed on 5-d-old hormone-starved cells (0.0 cells) for 2 h. Addition of the various inducers was by injection of concentrated stock solutions into the Erlenmeyer flasks. Chemicals used were from Merck (Darmstadt, Germany) except ACC and SA, which were a kind gift from the Bulb Research Centre (Lisse, The Netherlands). FC was provided by Dr. A.H. de Boer (University of Groningen). Furthermore, we are grateful to Dr. J. Memelink (University of Leiden) for supplying us with the chitinase, the β-1-3-glucanase, the acidic PR-1, and the pCNT6 cDNAs. The soybean histone H4 cDNA clone was a kind gift from Dr. H. Franssen (Agricultural University, Wageningen).

RESULTS

Accumulation of pCNT mRNAs during the Growth Cycle of a Tobacco Cell-Suspension Culture

The tobacco cell-suspension culture used in the present experiments (LT 0.05) has been extensively characterized (De Gunst et al., 1990). Several population growth features of the culture are relevant: upon transfer of early-stationary-phase cells to fresh medium containing 0.22 μM 2,4-D, cells are quickly reactivated to pass through the first cell cycle. The observed lag phase of about 26 h represents the non-mitotic part of the first cell cycle. The cell population is almost doubled 30 h after transfer. The initial synchronous wave of cell divisions rapidly decays because of variation in cycle times. The growth of the cell population stops at about 95 h after transfer, when cells are progressively leaving the cell cycle (De Gunst et al., 1990). To delimit the S-phase of the first cell cycle, we have used a cDNA clone corresponding to a soybean histone H4 mRNA that is regarded as an S-phase marker (Heintz, 1991). Figure 1 shows that the histone mRNA level increased rapidly between 6 and 11 h after transfer. The signal was detectable until approximately 95 h, which is about the time cells were progressively leaving the cell cycle. No clear modulations of H4 mRNA could be detected during the exponential phase of cell growth. In all probability, this is the result of the rapid decay of the initial synchrony.

The patterns of accumulation of pCNT103, -107, -114, and -115 mRNA during the growth cycle were similar but different from that of the histone H4 mRNA (Fig. 2). Starting about 30 min after transfer, the mRNA levels rapidly increased, reaching maximum levels at 4 h for pCNT114 and at 4 to 6 h for pCNT103 and -107. The mRNA corresponding to pCNT115 reached its maximum level 1.5 h after transfer. Subsequently, the mRNA levels decreased and reached their low initial levels at about 11 h after transfer. During the subsequent phases of the growth cycle, the level of the mRNAs corresponding to the four cDNA clones remained low (shown only for pCNT103 in Fig. 1).

To investigate transcriptional activity of the genes encoding the four classes of mRNAs, nuclear run-on experiments were performed. Nuclei were isolated from cells at 0, 1, 2, 4, 8,

and 24 h after transfer. Figure 3 shows that transcriptional activity of the genes encoding pCNT103, -107, and -114 mRNA corresponded with the accumulation of the mRNAs. We could not detect any significant transcriptional activity of the gene encoding pCNT115 mRNA. If this reflects the transcriptional activity of the pCNT115 gene in vivo, the accumulation of its mRNA might be due to posttranscriptional regulation.

The transcriptional rate of the control clone (GUS) also showed a distinct variation, reaching maximum levels approximately 4 h after transfer. This is in good agreement with recently published data that indicate that the TR1′ promoter is also under positive auxin regulation (Langridge et al., 1989; Saito et al., 1991).

The transient accumulation of the four mRNAs was probably not an effect of transfer of cells to fresh medium. If cells were transferred to fresh medium lacking 2,4-D, the mRNA levels were substantially lower than in cells transferred to medium containing 2,4-D (Fig. 4). Previously we found that early stationary-phase cells that were transferred to fresh medium lacking 2,4-D are still capable of a limited number of cell divisions (Van Der Zaal et al., 1987b). We have demonstrated that early-stationary-phase cells have stored 2,4-D, which is released into the medium upon transfer (K. Koens, unpublished results). We believe that it is this 2,4-D that triggered the moderate mRNA accumulation and the limited number of cell divisions when cells were transferred to fresh medium lacking 2,4-D.

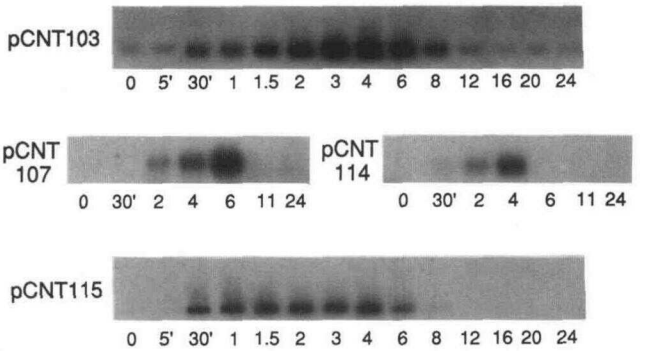

Figure 2. Modulation during the first 24 h of the expression of four 2,4-D-induced genes after the transfer of stationary cells to fresh medium with 0.22 μM 2,4-D. Northern blots of RNA samples (20 μg) taken at the indicated times (in h) were hybridized with plasmids containing the cDNAs of pCNT103, -107, -114, and -115.

Possible Modulation of Sensitivity of Cells toward 2,4-D upon Transfer to Fresh Medium

We have demonstrated in the preceding section that the mRNAs corresponding to the cDNA clones pCNT103, -107, -114, and -115 show a transient accumulation before the onset of DNA synthesis in cells passing through the first cell cycle. The next set of experiments was performed to determine whether the down-regulation of the mRNAs might be connected to desensitization of the cells to auxin. Eighteen hours after cells transferred to 0.05 medium, the pCNT103 mRNA was down-regulated to its initial level. We then injected a final concentration of 0.22 μm of 2,4-D into the medium. This treatment did not result in a significant increase in the mRNA level (Fig. 5). However, if we injected a concentration of 2,4-D 5 or 10 times higher, giving a final concentration of 1.1 or 2.2 μM, the mRNA increased substantially. This could mean that the cells were desensitized to auxin. Additional experiments are, however, necessary to confirm these preliminary results. Whether this desensitization is restricted to a particular stage of the cell cycle remains to be investigated. It is interesting that Dominov et al. (1992) observed the same desensitization reaction with another auxin-induced gene, pLS216, in *Nicotiana plumbaginifolia* suspension cultures.

Analysis of 103 mRNA Sense- and Antisense-Overexpressing Cell Lines

Two cell lines were derived from tobacco plants transformed with constructs containing the cauliflower mosaic virus 35S promoter and the pCNT103 coding region in either sense or antisense orientation. The cells were grown under the same conditions as LT 0.05, except for the concentration of 2,4-D, which was 2.2 μM. This concentration was used because young cell cultures metabolize 2,4-D more rapidly than well-established cultures such as LT 0.05 (our unpublished results). The transgenic cultures had about the same

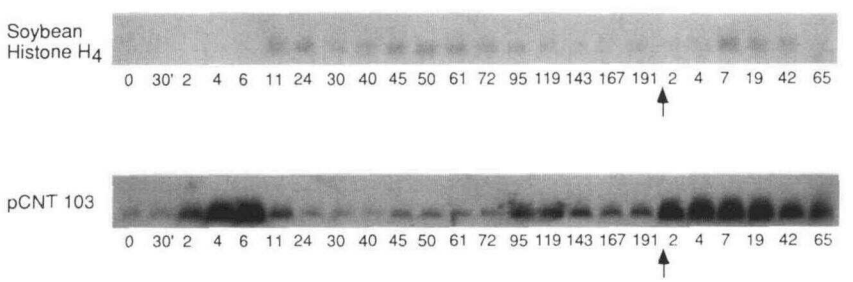

Figure 1. Modulation of the expression of a 2,4-D-induced mRNA (pCNT103) and of a soybean histone H4 mRNA during the growth cycle of a tobacco cell-suspension culture. From a tobacco cell-suspension culture grown in a fermenter on medium containing 0.22 μM 2,4-D, samples were taken at the indicated times (h). RNA blots (20 μg per lane) were hybridized with ^{32}P-labeled plasmids as indicated. After 191 h, additional 2,4-D was added to the culture to a final concentration of 2.2 μM (indicated by arrows).

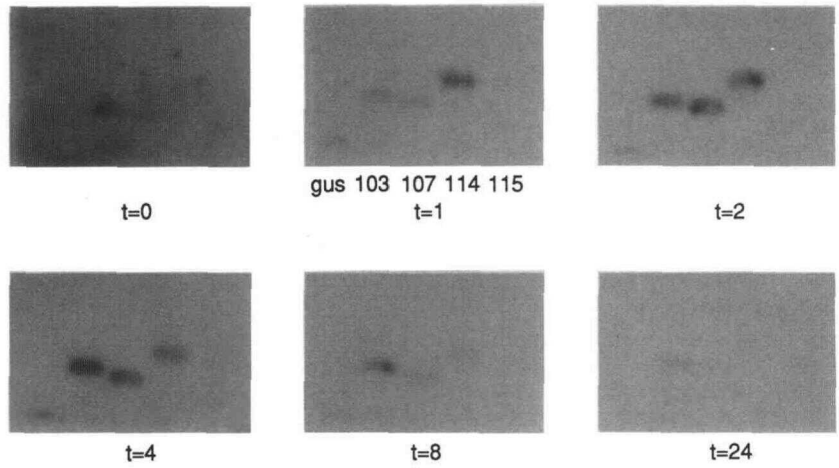

Figure 3. In vitro transcription studies. Nuclei were isolated from cells at 0, 1, 2, 4, 8, and 24 h after transfer of stationary cells to new medium containing 0.22 μM 2,4-D. After in vitro RNA synthesis, the labeled transcripts (3×10^7 cpm) were hybridized to Southern blots of *Eco*RI-digested plasmid DNA of pMPHH10MK (GUS), pCNT103 (103), pCNT107 (107), pCNT114 (114), and pCNT115 (115).

doubling time (about 30 h) as LT 0.05 and could be kept at a 7-d growth cycle. Figure 6 shows the level of pCNT103 mRNA during the first 24 h after transfer of the sense (27 series) and antisense cell (13 series) lines to fresh medium containing 2.2 μM 2,4-D. In the sense line, we observed a slight modulation of pCNT103 mRNA at a relatively high level, whereas in the antisense lines, pCNT103 mRNA stayed at an extremely low level.

To demonstrate that the altered levels of pCNT103 mRNA in the transgenic cell lines were specific, the expression of the homologous gene encoding pCNT107 mRNA (54% homology at the nucleotide level with the coding region of pCNT103) was examined using the same northern blots. Figure 6 shows that pCNT107 mRNA was not suppressed in the antisense line. Figure 6 also shows the levels of pCNT103 mRNA and pCNT107 mRNA in LT 0.05. The pattern of pCNT107 mRNA expression in the sense and antisense lines and in LT 0.05 was almost the same. The higher levels of pCNT107 mRNA in the sense and antisense lines at 11 h and to a lesser extent at 24 h, as compared to the levels in the LT 0.05 line, might be due to the higher 2,4-D concentration in the medium of these cell lines.

In a next set of experiments, the antisense and LT 0.05 cell lines were depleted of 2,4-D by growing them for 5 d in 2,4-D-free medium. Subsequently, 2,4-D was injected into the culture medium, giving a final concentration of 2.2 μM. This treatment brought about renewed cell division activity in both cell lines. In the antisense cell line, this treatment did not induce pCNT103 mRNA accumulation, whereas it did in the LT 0.05 cell line (Fig. 6).

Induction of pCNT103, -107, and -114 mRNA by Nonmitogenic Stimuli

When we analyzed the sequence data of cDNAs and genomic clones of the pCNT103, -107, and -114 gene families, we found significant homologies with a few other genes (Van Der Zaal et al., 1991). For example, pCNT103 has a high homology with Gmhsp26-A, a cDNA isolated from soybean seedlings. The gene corresponding to Gmhsp26-A encodes a heat-shock protein. This gene can also be induced by auxins, ABA, and some heavy metals (Czarnecka et al., 1984, 1988). Gmhsp26-A is identical with pGH2–4, a cDNA clone corresponding to mRNA species in soybean hypocotyls that are induced by auxin (2,4-D) but not by heat shock (Hagen et al., 1988). This prompted us to study in detail the specificity of induction of the pCNT103, -107, and -114 mRNAs. We first subjected 5-d-old auxin-starved LT 0.05 cells to heat shock, cold shock, and to some heavy metals. All treatments were for 2 h.

Figure 7 shows that neither cold nor heat shock significantly induced accumulation of the pCNT103 and -114 mRNAs, but the level of pCNT107 was affected by these treatments. Addition of Ag^+, Zn^{2+}, Cu^{2+}, or Co^{2+} (all at 0.1 mM) did not substantially induce accumulation of pCNT103 and -107 mRNA, but Ag^+ and Cu^{2+} did induce accumulation of pCNT114 mRNA to nearly the same extent as 2.2 μM 2,4-D.

Next, we tried some other treatments, including addition of ACC, the precursor of ethylene, SA, which is thought to be one of the naturally occurring inducers of PR proteins

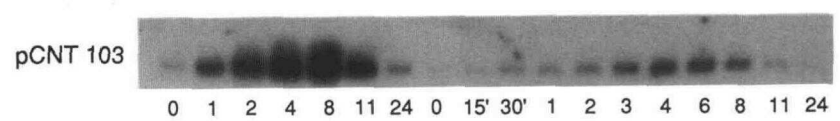

Figure 4. Comparison of the expression of pCNT103 mRNA after transferring cells to medium with 2,4-D or to hormone-free medium. Samples were taken at the times indicated from stationary-phase tobacco cells transferred to either 0.22 μM 2,4-D-containing medium (left) or to 2,4-D-free medium (right). The RNA blot was hybridized with ^{32}P-labeled pCNT103 plasmid DNA.

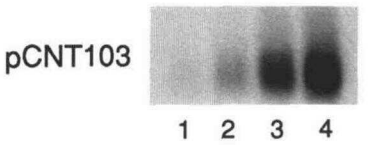

Figure 5. Comparison of the expression of pCNT103 mRNA 18 h after the transfer of cells (to medium with 0.22 μM 2,4-D) and treatment for an additional 3 h with H_2O (lane 1), 0.22 μM 2,4-D (lane 2), 1.1 μM 2,4-D (lane 3), or 2.2 μM 2,4-D (lane 4).

(Malamy et al., 1990), and FC, which is a fungal toxin capable of mimicking some of the effects of auxin (Marrè, 1977). Figure 8 shows that SA (0.1 mM) induced a substantial accumulation of all three mRNA species. ACC had some effect but only at high concentrations (10 mM), whereas FC (1 μM) had no effect.

Unlike 2,4-D, SA did not induce renewed cell-division activity in our cell lines (data not shown).

Modulation of Expression of Some PR Genes in LT 0.05

Because we found that SA is a relatively strong inducer of pCNT103, -107, and -114 mRNA, and because these genes showed considerable homology with the PR protein prp1 from potato (Taylor et al., 1990), we compared the expression pattern of some PR genes and pCNT103, -107, and -114 in the LT 0.05 suspension culture. We selected a few well-known PR genes for which cDNA clones were available, i.e. cDNAs coding for a basic form of a chitinase, a basic β-1,3-glucanase, and an acidic PR-1. These cDNAs were all isolated from tobacco (Memelink et al., 1990). To study the expression of these PR genes, the same northern blots as shown in Figure 1 were hybridized with the corresponding cDNAs. Figure 9 shows that the mRNA of the basic chitinase gene was relatively high in stationary-phase cells. A sharp decline of the mRNA occurred between 11 and 24 h after transfer of cells to fresh medium containing 0.22 μM 2,4-D. During the exponential growth phase, the mRNA level remained low. However, from about 95 h, when cells were progressively leaving the cell cycle, the mRNA increased and stayed at a relatively high level during the stationary phase. The β-1–3-glucanase mRNA levels showed a pattern similar to that of the basic chitinase, whereas PR-1 mRNA could not be detected at any stage of the growth cycle (data not shown). The mRNA level of a control clone, pCNT6, remained unchanged during the entire growth cycle, except for a short period between 11 and 45 h after transfer, during which the mRNA level was somewhat decreased.

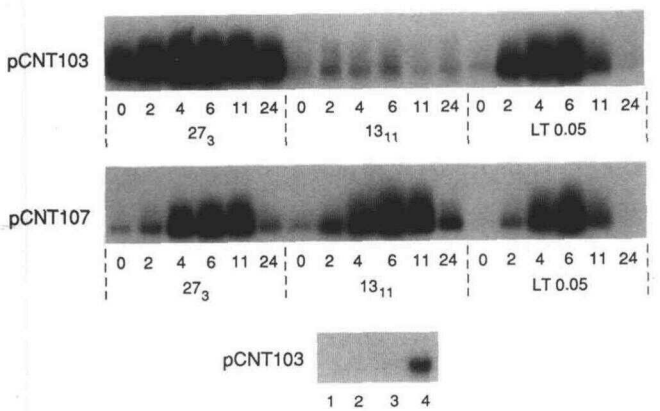

Figure 6. Effect of overexpression of sense or antisense 103 mRNA on the accumulation of pCNT103 mRNA. Cell-suspension cultures derived from transgenic sense (27_3) or antisense (13_{11}) 103 mRNA-producing tobacco plants grown on 2.2 μM 2,4-D medium were transferred to fresh medium with the same 2,4-D concentration. Northern blots were hybridized with plasmids containing pCNT103 (upper panel) and 107 (middle panel) cDNAs. As a control, RNA from untransformed tobacco cell-suspension cultures (LT 0.05) grown on 0.22 μM 2,4-D was used. In the lower panel, a northern blot of total RNA samples from 5-d-old hormone-starved transgenic antisense (13_{11}) 103 mRNA-producing cells (lanes 1 and 2) or from hormone-starved untransformed control cells (lanes 3 and 4) was hybridized with pCNT103 plasmid DNA. Cells were treated for 2 h with water (lanes 1 and 3) or 2.2 μM 2,4-D (lanes 2 and 4).

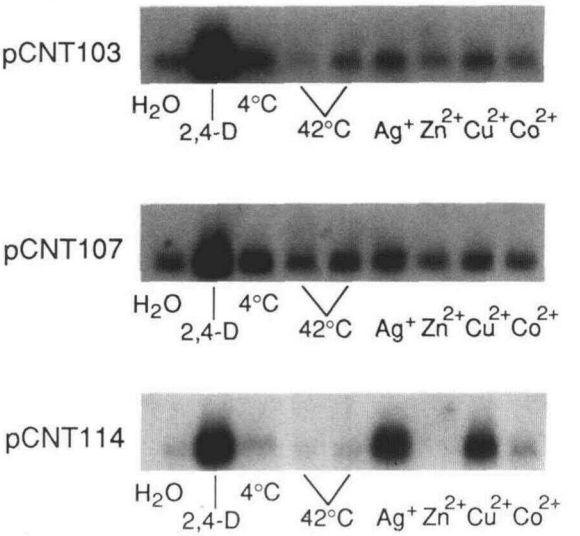

Figure 7. Effect of various stress treatments on induction of pCNT103, -107, and -114 mRNAs. Northern blots of total RNA samples from 5-d-old hormone-starved cells treated for 2 h with H_2O, 2.2 μM 2,4-D, or 10 mM $AgNO_3$, $ZnCl_2$, $CuSO_4$, or $CoCl_2$, respectively (final concentrations). The heat shock was performed by placing the tobacco cells at 42°C for 15 min or 2 h, respectively; the cold shock was done by placing the cells for 2 h at 4°C.

Figure 9 shows that readdition of 2,4-D (final concentration of 2.2 μM) after 191 h did not affect the basic chitinase mRNA and pCNT6 mRNA level as measured during a period of about 65 h. The 2,4-D treatment of stationary-phase cells at this time did, however, bring about an increase of pCNT103, -107, -114, and histone H4 mRNA (shown only for pCNT103 and histone H4 in Fig. 1).

We conclude that unlike the genes encoding pCNT103, -107, and -114 mRNA, which are probably more directly regulated by 2,4-D, the PR genes encoding basic chitinase and β-1–3-glucanase are developmentally regulated. The PR genes were expressed in cells that left the cell cycle and started to differentiate into parenchymatous cells at about 95

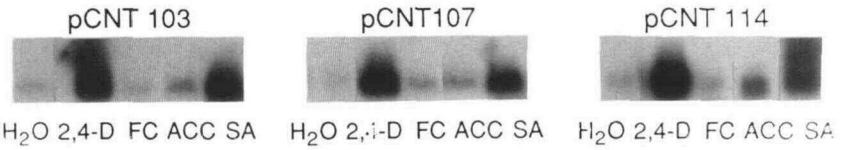

Figure 8. Effect of some other factors on the mRNA induction of three 2,4-D-induced genes. Northern blots from RNA samples from 5-d-old hormone-starved tobacco cell-suspension cultures treated for 2 h with H_2O, 2.2 μM 2,4-D, 1 μM FC, 10 mM ACC, or 0.1 mM SA (final concentrations).

h after transfer of LT 0.05 cells to fresh medium. The PR genes used in this study are, therefore, excellent differentiation markers in the LT 0.05 cultures.

DISCUSSION

We have shown that genes encoding the pCNT103, -107, and -114 mRNAs are under positive transcriptional control by auxin and are transiently expressed during transition from G_0 to S phase of the cell cycle. Recently, Takahashi et al. (1989) reported the characterization of an auxin-induced gene named *parA* that was also expressed during transition from G_0 to S phase in tobacco mesophyll protoplasts. This *parA* gene is identical with our cDNA pCNT114 (our unpublished results). Takahashi et al. (1989) conclude from the expression pattern of the *parA* gene that its protein product might play a role in the initiation of meristematic activity in differentiated mesophyll cells. Induction of cell division activity in our system and cell elongation in other systems are auxin specific. The concomitant early induction of particular genes does not necessarily mean that these genes are essential for triggering these two processes. To date there is no evidence that the auxin-induced genes characterized thus far are essential parts of the signal transduction pathway leading to cell division or cell elongation.

Recently, we found that proteins encoded by the genes corresponding to pCNT103, -107, and -114 cDNAs show a limited but significant homology to both plant and animal GSTs. In addition, we have demonstrated in vitro GST activity of the 103 protein (F.N.J. Droog, unpublished data). Thus, we are probably dealing with a multigene family encoding GSTs. The reason for the existence of such a large gene family in tobacco, and probably other species encoding proteins with similar functions, is unknown. GSTs are involved in a variety of processes, such as detoxification reactions, protection against oxidative tissue damage, and intracellular transport but also in the production of secondary messengers such as prostaglandins and leukotrienes (Söderström et al., 1985; Ujihara et al., 1988). A role for these secondary messengers in higher plants remains to be determined. Recently, however, it was reported that auxins could induce phospholipase A activity in plant membranes (Andre and Scherer, 1991), which in animal systems leads to the production of arachidonic acid, a well-known regulator of leukotrienes. Although arachidonic acid does not occur in plant membranes, a homologous lipid-like linolenic acid could perhaps perform similar functions in plant cells to produce jasmonic acid as a second messenger.

A potential role for the GSTs in detoxifying 2,4-D is another possibility. GSTs are enzymes that catalyze the addition of GSH to a wide range of electrophilic substrates. Perhaps they can also transfer GSH to 2,4-D, thereby regulating the amounts of free 2,4-D in the cells.

The diverse functions of the GSTs make it difficult to exclude a role for pCNT103 in cell division. Specific suppression of the accumulation of pCNT103 mRNA did not affect cell division in the tobacco cell culture. This, however, does not necessarily prove that this protein is not required, because of the presence of related gene families. By analogy with animal systems, it seems likely that the different types of GST will fulfill different functions in plant cells. The role of GSTs in a normal cellular process like cell division remains an open question.

The various auxin-induced genes that have been isolated so far can be divided into two groups. First, the genes that are induced by a variety of stimuli and that show a high degree of homology, consisting of pCNT103, -107, -114; Gmhsp26A; pGH2–4; parA; parB; prp1; and pLS216 probably all belong to the superfamily of GSTs and are likely to be involved in adaptation responses. The genes belonging to the second group, such as the SAURs, pGH1 and pGH3,

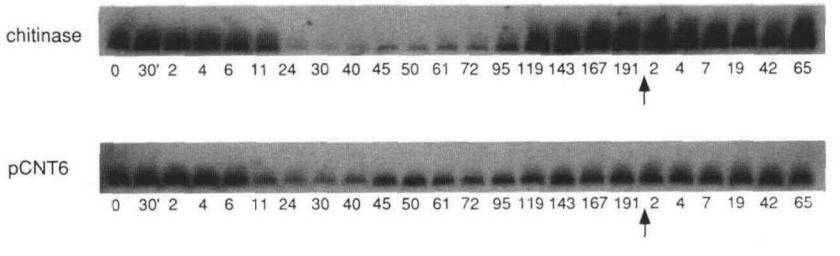

Figure 9. Modulation of the expression of a PR gene. The same northern blots used in Figure 1 were rehybridized with a plasmid containing the basic form of a chitinase cDNA isolated from tobacco and with a control clone, pCNT6, coding for a gene that is unaffected by auxins. After 191 h 2,4-D was added to a final concentration of 2.2 μM (indicated by arrows).

AUX22 and AUX28, and pIAA4/5 and pIAA6 are apparently induced only by auxins (based on other stimuli that have been tested) and do not show any significant homology with the first group (based on the sequences that have been determined). Functions for the encoded proteins of the second group have not yet been established.

A similar situation seems to occur with the ABA-induced genes. Most genes that are induced by this hormone are also induced by various other environmental and stress stimuli, such as wounding, salt stress, osmotic stress, and drought (Skriver and Mundy, 1990). In recent studies of mammalian growth factors, it was observed that, although the growth factors epidermal growth factor and nerve growth factor induce similar early responses, the eventual biological effect of these factors is entirely different, affecting cell division and neural differentiation, respectively. Although they have distinct receptors, epidermal growth factor and nerve growth factor trigger the same set of early responses, such as Tyr phosphorylation, activation of a Na^+/K^+ pump, protein phosphorylation, ion channel activation, and the induction of the same early genes (Chao, 1992). Thus, even in this well-studied case, the specificity of the two signals is far from clear.

An interesting question is whether the two types of auxin-induced genes are regulated by the same signal transduction pathway and whether different stimuli such as elicitors, SA, and heavy metals use the same or different pathways. Using the more precise knowledge of auxin-induced gene expression patterns and cell kinetics in tobacco cell cultures as reported in the present paper, we are now working on methods to discriminate between receptors and other transducing proteins involved in either auxin or SA and elicitor-induced promoter activity of the gene encoding pCNT103 mRNA. The activity of a variety of auxin analogs and the differential suppression of auxin-induced promoter activity by antibodies directed against the putative auxin-binding site will be reported elsewhere.

ACKNOWLEDGMENTS

We are indebted to J.J. Quintero for culturing tobacco cells. We wish to express our sincere thanks to Professor M.A. Hall (University of Wales, Aberystwyth, UK) and R. Libbenga for their critical reading of the manuscript.

Received November 30, 1992; accepted February 25, 1993.

LITERATURE CITED

Ainley WM, Walker JC, Nagao RT, Key JL (1988) Sequence and characterization of two auxin-regulated genes from soybean. J Biol Chem **263:** 10658–10666

Andre B, Scherer GFE (1991) Stimulation by auxin of phospholipase A in membrane vesicles from an auxin-sensitive tissue is mediated by an auxin receptor. Planta **185:** 209–214

Brummell DA, Hall JL (1987) Rapid cellular responses to auxin and the regulation of growth. Plant Cell Environ **10:** 523–543

Chao MS (1992) Growth factor signaling: where is the specificity? Cell **68:** 995–997

Czarnecka E, Edelman L, Schoffl E, Key JL (1984) Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. Plant Mol Biol **3:** 45–58

Czarnecka E, Nagao RT, Key JL, Gurley WB (1988) Characterization of Gmhsp26-A, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol Cell Biol **8:** 1113–1122

De Gunst MCM, Harkes PAA, Val J, Van Zwet WR, Libbenga KR (1990) Modelling the growth of a batch culture of plant cells: a corpuscular approach. Enzyme Microb Technol **12:** 61–71

Dominov JA, Stenzier L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell **4:** 451–461

Felle H, Peters W, Palme K (1991) The electrical response of maize to auxins. Biochim Biophys Acta **1064:** 199–204

Guilfoyle TJ (1986) Auxin-regulated gene expression in higher plants. CRC Crit Rev Plant Sci **4:** 247–277

Hagen G, Guilfoyle T (1985) Rapid induction of selective transcription by auxins. Mol 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

Hagen G, Uhrhammer N, Guilfoyle T (1988) Regulation of expression of an auxin-induced soybean sequence by cadmium. J Biol Chem **263:** 6442–6446

Heintz N (1991) The regulation of histone gene expression during the cell cycle. Biochim Biophys Acta **1088:** 327–339

Key JL (1989) Modulation of gene expression by auxin. Bioessays **11:** 52–58

Langridge WHR, Fitzgerald KJ, Koncz C, Schell J, Szalay AA (1989) Dual promoter of *Agrobacterium tumefaciens* mannopine synthase genes is regulated by plant growth hormones. Proc Natl Acad Sci USA **86:** 3219–3223

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant **8:** 100–127

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250:** 1002–1004

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Marrè E (1977) Fusicoccin: a tool in plant physiology. Annu Rev Plant Physiol **30:** 273–288

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Mol Biol **9:** 611–623

Memelink J, Linthorst HJM, Schilperoort RA, Hoge JHC (1990) Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol Biol **14:** 119–126

Mennes AM, Bouwman H, Van Der Burg MPM, Libbenga KR (1978) RNA synthesis in isolated tobacco callus nuclei, and the influence of phytohormones. Plant Sci Lett **13:** 329–339

Peters WS, Felle H (1991) Control of apoplast pH in corn coleoptile segments. II. The effects of various auxins and auxin analogues. J Plant Physiol **137:** 691–696

Saito K, Yamazaki M, Kaneko H, Murakoshi I, Fukuda Y, Van Montagu M (1991) Tissue-specific and stress-enhancing expression of the TR promoter for mannopine synthase in transgenic medicinal plants. Planta **184:** 40–46

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512

Söderström M, Mannervik B, Örning L, Hammarström S (1985) Leukotriene C4 formation by three distinct forms of human cytosolic glutathione transferase. Biochem Biophys Res Commun **128:** 265–270

Takahashi Y, Kuroda H, Tanaka T, Machida Y, Takebe I, Nagata T (1989) Isolation of an auxin-regulated gene cDNA expressed during the transition from G_0 to S phase in tobacco mesophyll protoplasts. Proc Natl Acad Sci USA **86:** 9279–9283

Taylor JL, Fritzemeier KH, Häuser I, Kombrink E, Rohwer F, Schröder M, Strittmatter G, Hahlbrock K (1990) Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato. Mol Plant Microbe Interact **3:** 72–77

Theologis A (1986) Rapid gene regulation by auxin. Annu Rev Plant Physiol **37:** 407–438

Theologis A, Huynh TV, Davis RW (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. J Mol Biol **183:** 53–68

Ujihara M, Tsuchida S, Satoh K, Urade Y (1988) Biochemical and immunological demonstration of prostaglandin D2, E2 and F2 formation from prostaglandin H2 by various rat glutathione S-transferase isozymes. Arch Biochem Biophys **264:** 428–437

Van Der Zaal EJ (1989) Auxin-induced gene expression in tobacco. PhD Thesis. Rijksuniversiteit Leiden, The Netherlands

Van Der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible genes from tobacco can lead to auxin-inducible and root tip-specific expression. Plant Mol Biol **16:** 983–998

Van Der Zaal EJ, Memelink J, Mennes AM, Quint A, Libbenga KR (1987a) Auxin-induced mRNA species in tobacco cell cultures. Plant Mol Biol **10:** 145–157

Van Der Zaal EJ, Mennes AM, Libbenga KR (1987b) Auxin-induced rapid changes in translatable mRNAs in tobacco cell suspension. Planta **172:** 514–519

Van Slogteren GMS, Hoge JHC, Hooykaas PJJ, Schilperoort RA (1983) Clonal analysis of heterogeneous crown gall tumor tissues induces by wild-type and shooter mutant strains of *Agrobacterium tumefaciens*—expression of T-DNA genes. Plant Mol Biol **2:** 321–333