The EMBO Journal vol.6 no.12 pp.3579–3583, 1987

Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco

J.Memelink, J.H.C.Hoge and R.A.Schilperoort

Molbas Research Group, Department of Plant Molecular Biology, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

Communicated by W.Möller

Tobacco shoots exposed to elevated endogenous or exogenous cytokinin levels are unable to develop roots and lack apical dominance. We have isolated cDNA copies of five mRNA species that accumulate to elevated levels in such cytokinin-stressed shoots via differential screening of a cDNA library of transgenic shoots which contain an active T-DNA cytokinin gene (*T-cyt* gene) from *Agrobacterium tumefaciens*. Four of the cDNA clones were found to correspond to plant defence-related mRNAs, encoding extensin, chitinase, PR-1 and a PR-1-like protein, respectively. In normal tobacco plants PR-1 mRNA is relatively rare in all organs. The other four mRNAs occur at relatively low levels in shoots, especially in leaves, but are very prevalent in roots. Extensin mRNA, for example, is not detectable in leaves, while it is an abundant mRNA in roots and stems. In normal shoots cultured on cytokinin-containing medium all five mRNAs accumulate to elevated levels, similar to those found in transgenic *T-cyt* shoots. We conclude that the imposed cytokinin stress causes changes in the tissue-specific control of the levels of several defence-related mRNA species in tobacco.

Key words: cytokinins/defence-related mRNAs/gene regulation/T-DNA/tobacco

Introduction

Plant growth and differentiation is greatly influenced by several classes of phytohormones. A classical example is the control of organogenesis in tissue-cultured tobacco by auxins and cytokinins (Skoog and Miller, 1957). Relatively high cytokinin levels in the culture medium induce shoot formation, but suppress root formation, whereas relatively high auxin levels have opposite effects. These phytohormones are widely applied in plant tissue culture and there is a large body of literature concerning their effects on plant morphology. However, at present very little is known about their mode of action. In analogy to animal systems (for a review, see Anderson, 1984), early responses to phytohormone application are thought to involve specific receptor proteins, that become activated and alter the expression of primary target genes. Their gene products regulate other genes and eventually lead to a cascade of secondary effects.

Most reports on plant gene expression in response to the application of hormones concern auxins (for a review, see Theologis, 1986). There exist only very limited data concerning cytokinin-mediated changes in mRNA populations. Among the latter are enhanced accumulation of nuclear-encoded mRNAs for chloroplast proteins in tobacco suspension culture (Teyssendier de la Serve *et al.*, 1985) and *Lemna gibba* (Flores and Tobin, 1986), and reduction of chitinase (Shinshi *et al.*, 1987) and β-1,3-glucanase (Mohnen *et al.*, 1985) mRNA levels in tobacco callus tissue. All experimental systems studied to date with respect to hormone effects on plant gene expression make use of exogenously added hormones, and consequently contain uncontrolled variables related to uptake and transport of these compounds.

Alternative model systems for studies on the molecular basis of plant morphogenesis are provided by plant cells transformed by the T-DNA from the Ti-plasmid of the soil bacterium *Agrobacterium tumefaciens*. Two loci on the transferred T-DNA, the auxin locus and the cytokinin locus (*T-cyt* gene), code for the overproduction of auxins and cytokinins within each T-DNA transformed plant cell (for a review, see Morris, 1986). Infection of tobacco with *A.tumefaciens* induces undifferentiated, tumorous outgrowths when both loci are active. If only the auxin locus is active the induced tumours produce roots and if only the *T-cyt* gene is active shoots are produced. *In vitro* cultured transgenic tobacco shoots containing an active *T-cyt* gene, but not the auxin locus, lack apical dominance (reduced length, adventitious sprouts) and are unable to form roots (Memelink *et al.*, 1983; Peerbolte, 1986; Peerbolte *et al.*, 1987) as a result of *T-cyt* gene-directed cytokinin overproduction.

In search of plant genes which are (in-)directly regulated by the high endogenous cytokinin level and/or are involved in the establishment or maintenance of the aberrant, rootless phenotype, we screened a cDNA library of transgenic *T-cyt* shoots by differential hybridization. Here we describe the isolation and characterization of cDNA copies of five mRNA species with elevated levels in shoots with a cytokinin-imposed aberrant phenotype. Using these cDNA clones as probes, we determined the distribution of the corresponding mRNAs in transgenic *T-cyt* shoots and in normal tobacco plants.

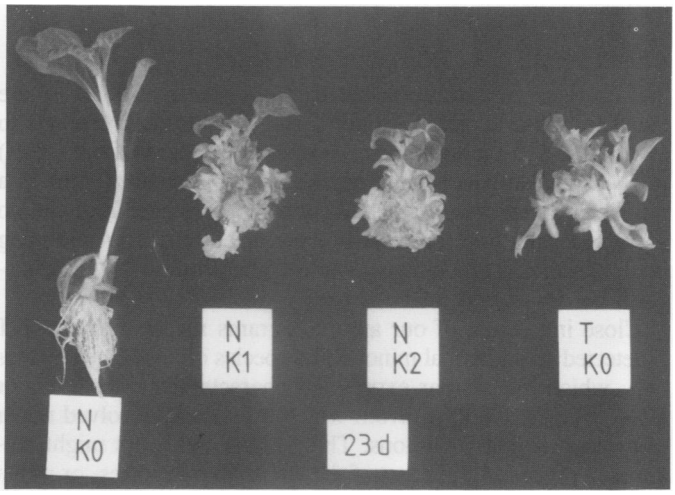

Fig. 1. Normal and cytokinin-imposed phenotypes. Normal SNT1016 shoots (**N**) were subcultured for 23 days on medium containing 0 (**K0**), 1 (**K1**) or 2 (**K2**) mg/l kinetin and *T-cyt* transformed SNT1005 (**T**) shoots were grown on hormone-free medium (**K0**).

Table I. Characteristics of selected cDNA clones

cDNA clone	Insert size (bp)	mRNA size (nt)	RTA[a] in transformed leaves	RLT[b]: transformed leaves versus normal leaves	RTL[b]: transformed leaves versus normal roots	Characteristics
pCNT 1	1350	1350	1.3	>100	1	Extensin
2	600	650	0.3	8	2	
3	700	900	2.2	25	>100	PR-1
4	550	1300	0.9	10	0.8	chitinase
5	800	950	0.6	10	0.8	PR-1-like
6	800	900	1	1.5	2	control
7	850	950	2.4	0.9	>100	rbcS

[a]Relative transcript abundance (RTA) is an estimation of mRNA abundance in transformed leaves and represents the number of molecules of each mRNA relative to the arbitrarily chosen reference mRNA 6.
[b]Relative transcript level (RTL) compares the levels of each mRNA in different organs as indicated in the headings.

Results

Isolation of cDNA clones

To isolate cDNA clones of mRNAs that accumulate in tobacco shoots with a cytokinin-imposed phenotype, we used axenic cultures of *T-cyt* transformed, rootless SNT1005 shoots and normal SNT1016 plants (Figure 1). Both cultures were obtained by vegetative propagation of individual seedlings derived from sexual crosses between grafted *T-cyt* transformed SR1-4013-3 shoots and normal SR1 tobacco plants. One half of the seedlings originating from such crosses are normal, SNT1016-like, and the other half are T-DNA transformed, SNT1005-like (Peerbolte, 1986; Peerbolte *et al.*, 1987). Transformed seedlings initially produce thick primary roots, but after 2–3 weeks further root development is impaired and roots degenerate to callus tissue (Memelink *et al.*, 1983; Peerbolte, 1986; Peerbolte *et al.*, 1987).

A cDNA plasmid library of total SNT1005 shoots was constructed and differentially screened. Replica filters containing 480 independent transformants were hybridized to radioactive cDNAs synthesized on SNT1005 and SNT1016 poly(A) RNA. Recombinant plasmids were isolated from transformants that hybridized more strongly to the SNT1005 than to the SNT1016 cDNA probe, and from some transformants showing equal hybridization to both probes. Characterization of the cDNA inserts by restriction enzyme analysis, cross-hybridization and Northern blot hybridization to SNT1005 and SNT1016 total RNAs led to the selection of six cDNA clones, pCNT 1–6, for further studies.

Characterization of selected cDNA clones

The characteristics of the selected cDNA clones pCNT 1–6 are listed in Table I. cDNA clone pCNT 7, which corresponds to a ribulose-1,5-biphosphate-carboxylase small subunit (rbcS) mRNA, is also listed, since it was used as a control clone in a number of experiments. cDNA insert sizes range from 550 to 1350 basepairs and the cDNAs hybridized to mRNAs ranging in size from 650 to 1350 nucleotides. These mRNAs will henceforth be designated mRNAs 1–7.

Close inspection of our autoradiograms revealed that pCNT 5 detected an additional minor RNA species of ~750 nucleotides (nt), which has similar expression characteristics to the major RNA species of 950 nt, from which it is poorly resolved under our experimental conditions. This smaller transcript might represent a processing product of the larger RNA species, or might be transcribed from a related coordinately regulated gene.

pCNT 6 corresponds to an unidentified mRNA, which is expressed to similar levels in all tissues analysed. These include different plant organs, T-DNA transformed tissues and tissues treated with hormones. Therefore pCNT 6 was used throughout this study as a hybridization control clone to confirm that equal amounts of mRNA were present in all lanes of RNA gel blots.

Genes that are induced by pathogenic infection, such as chitinase (Shinshi *et al.*, 1987), β-1,3-glucanase (Mohnen *et al.*, 1985) or PR-1 (Antoniw *et al.*, 1981, 1983) were reported to be expressed at elevated levels in axenically cultured tobacco tissues and were suggested to be regulated by cytokinins and auxins. Therefore, we cross-hybridized our cDNA clones to tobacco cDNA clones A–F of mRNAs that are induced in leaves by infection with tobacco mosaic virus (Hooft van Huijsduijnen *et al.*, 1986). pCNT 3 hybridized to clone B, which corresponds to an mRNA encoding the pathogenesis-related protein PR-1[b]. pCNT 4 hybridized to clone F, corresponding to an mRNA encoding chitinase (Hooft van Huijsduijnen *et al.*, 1987). Its sequence proved to be identical to 550 nt of the 3′ end of a tobacco chitinase cDNA sequence published recently by Shinshi *et al.* (1987). pCNT 5 showed weak cross-hybridization to clone B and pCNT 3. Its nucleotide sequence has 60% homology to the PR-1 sequence and specifies a mature protein of 173 amino acids. The N-terminal part of 137 amino acids of this protein has 67% homology to the PR-1[b] amino acid sequence (Cornelissen *et al.*, 1987). However, pCNT 5 did not detect PR-1 mRNAs, and pCNT 3 did not detect mRNA(s) 5, at the stringency of hybridization and exposure times that we applied in our experiments. Sequence data obtained so far indicate that pCNT 1 corresponds to an mRNA encoding extensin, which is a hydroxyproline-rich cell wall glycoprotein. The predicted amino acid sequence contains the characteristic repeating ser–$(pro)_4$ unit, and the putative signal peptide is nearly identical to a published carrot extensin signal peptide (Chen and Varner, 1985).

mRNA accumulation of a result of endogenous overproduction of cytokinins

Gel blots containing RNAs from total SNT1005 shoots and total SNT1016 plants and from 2-week-old normal and *T-cyt* transformed seedlings (~500 pooled individuals each) were hybridized to pCNT 1–6 (Figure 2). pCNT 1, 2, 4 and 5 hybridized 8- to 2-fold more strongly to SNT1005 RNA than to SNT1016 RNA, while pCNT 3 hybridized >100-fold more strongly to SNT1005 RNA. In the case of 2-week-old seedling RNAs, differential expression of mRNAs corresponding to pCNT 1–5 was even more pronounced. This indicates that mRNAs 1–5 are preferentially expressed in *T-cyt* transformed seedlings, in spite of the fact that all seeds were derived from the same sexual cross and germinated under identical conditions in one Petri dish. Furthermore, these data show that the observed expression characteristics are not specific for the SNT1005 shoot culture and the SNT1016 plant culture used to isolate cDNA clones, but are shared by a significant proportion of the total seedling population.

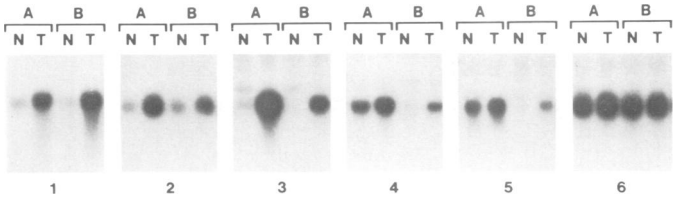

Fig. 2. mRNA 1–6 accumulation as a result of endogenous overproduction of cytokinins. Gel blots containing RNA from (**A**) established cultures of total SNT1005 shoots (**T**) and SNT1016 plants (**N**) and (**B**) from 500 pooled transformed (**T**) and 500 pooled normal (**N**) seedlings harvested 2 weeks after germination, were hybridized to cDNA clones pCNT 1–6.

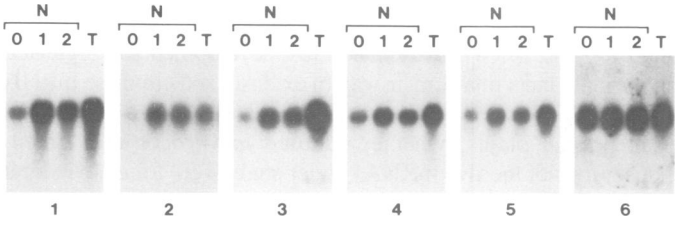

Fig. 3. mRNA 1–6 accumulation as a result of exogenously added cytokinins. Gel blots containing RNA from normal SNT1016 shoots (**N**), grown for 3 weeks on 0, 1 or 2 mg/l kinetin, and from transformed SNT1005 shoots (**T**), grown on hormone-free medium, were hybridized to cDNA clones pCNT 1–6. Kinetin concentrations are indicated at the top of the figure. Roots of normal plants grown on hormone-free medium were removed prior to RNA isolation.

As these and following hybridization experiments were done with total RNA preparations, they do not take into account possible variations in mRNA levels relative to rRNA. However, the constant hybridization levels obtained with the control clone pCNT 6 in all experiments indicate that the ratio of mRNA to rRNA must be fairly constant in all tissues studied.

mRNA accumulation as a result of exogenously added cytokinins

To confirm that the observed mRNA accumulation in *T-cyt* transgenic shoots is correlated with the cytokinin-imposed phenotype, normal SNT1016 shoots were cultured for 3 weeks on medium containing 1 or 2 mg/l of the synthetic cytokinin kinetin. On these media, SNT1016 shoots form rootless structures that are very similar to the *T-cyt* transformed SNT1005 shoots grown on hormone-free medium (Figure 1). mRNA 1–6 levels were determined in these shoots by Northern blot hybridization. In normal shoots grown on cytokinin-containing medium, mRNAs 1–5 accumulate to levels that are comparable with those present in transformed shoots, while mRNA 6 levels are unaffected (Figure 3). The extents of induction and accumulation of these mRNAs were measured over the total mass of shoot tissues. These experiments therefore do not distinguish between a uniform induction of the genes under study throughout the entire shoot, or a more dramatic induction in specific cell types.

mRNA levels in different organs of in vitro grown plants

To study the expression characteristics of mRNAs 1–7 in more detail, gel blots containing RNAs extracted from different organs of SNT1005 and SNT1016 plants were hybridized to the selected cDNA clones (Figure 4). In stems and leaves of transformed shoots the levels of mRNAs 1–5 are much higher than in corresponding normal organs. mRNAs 1 and 2 are slightly more prevalent in callus tissue formed at the base of transformed shoots than in transformed leaves, while mRNAs 3, 4 and 5 are present at similar high levels in different parts of SNT1005 shoots. Based on cloning frequencies and relative hybridization signals, we estimate that each mRNA constitutes ~0.2–2% of the total mRNA mass in transformed leaves. Thus in transformed tissues these are all very prevalent mRNAs and ~20- to 200-fold more abundant than *T-cyt* mRNA.

In normal plants, we expected all five mRNAs to be present at relatively low levels throughout the whole plant. This appeared to be true for mRNA 3, which is barely detectable only in leaves. In striking contrast mRNAs 1, 2, 4 and 5 are very abundant in normal roots. The relative expression levels (RTL) of mRNAs 1–5 in transformed leaves compared with normal leaves and roots were determined by densitometric scanning of the autoradiograms reproduced in Figure 4 and are listed in Table I. In transformed leaves the levels of mRNAs 1, 2, 4 and 5 are comparable with those found in normal roots, while in normal leaves these levels are 8- to >100-fold lower. Thus, although these four mRNAs have different abundances varying over an ~10-fold range (Table I; RTA), and show variations in the extent of differential expression between organs (Table I; RTL), their overall expression characteristics are very similar. The most striking example of the observed phenomena is the expression pattern of mRNA 1. While this mRNA is not detectable at all in normal leaves it is present at >100-fold higher levels in both transformed leaves and normal roots. Leaf-specific photosynthetic processes appeared to function normally in transformed leaves, as the rbcS clone pCNT 7 hybridized equally well to transformed and normal leaf RNAs. In addition pCNT 6, which probably corresponds to a 'housekeeping' gene, gave approximately equal hybridization signals with RNAs from different organs.

mRNA levels in different organs of normal plants grown in soil

To investigate whether the observed expression characteristics in normal plants are due to *in vitro* culture conditions, the levels of mRNAs 1–6 in different organs of normal tobacco plants grown in soil in a greenhouse were monitored. These plants carried flowers and green fruits. In the vegetative organs the expression patterns and levels of all mRNAs under study proved to be similar to those in *in vitro* grown normal plants. mRNAs 1, 2, 4 and 5 are present predominantly in roots, and the levels of mRNA 3 are very low in all organs (Figure 5). Remarkably, mRNAs 1, 2, 4 and 5 are also present in flowers at much higher levels than in leaves. Thus in normal plants grown in a greenhouse the overall expression characteristics of mRNAs 1, 2, 4 and 5 are once again strikingly similar.

Discussion

We have isolated cDNA copies of five mRNAs with elevated levels in *T-cyt* transgenic shoots that are unable to form roots and lack apical dominance due to endogenous overproduction of cytokinins. These mRNAs accumulate to similar high levels when root development and apical dominance are suppressed in normal shoots by exogenously added cytokinins. Their enhanced expression in *T-cyt* shoots is most conspicuous when levels in leaves are compared. In normal leaves the mRNA levels are relatively low, for mRNAs 1 and 3 even at or below the limit of detection, while in transformed leaves their levels are quite high. Two mRNAs (1 and 3) are almost as abundant as rbcS mRNA (mRNA 7). We also isolated clones of two mRNAs (6 and 7) with virtually identical levels in transformed and normal leaves, which indicates that there is not a general, aspecific increase of mRNA levels in *T-cyt* transformed leaves.

By cross-hybridization and sequence comparisons we were able to identify four of the isolated clones as cDNA copies of extensin, chitinase, PR-1 and a gene closely related to PR-1. The corresponding mRNAs are induced by wounding (Chen and Varner, 1985) and/or by pathogenic infection (Showalter *et al.*, 1985;

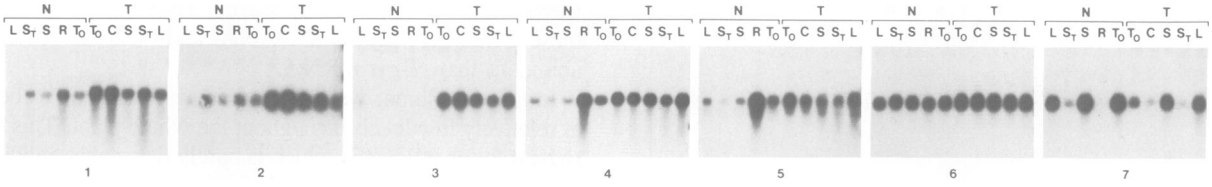

Fig. 4. Distribution of mRNAs 1–7 in normal SNT1016 plants (**N**) and *T-cyt* transformed SNT1005 shoots (**T**) grown *in vitro*. Gel blots containing RNA from total (T_0) plantlets, shoots (**S**), stems (S_T), leaves (**L**), roots (**R**) and callus (**C**) were hybridized to cDNA clones pCNT 1–7.

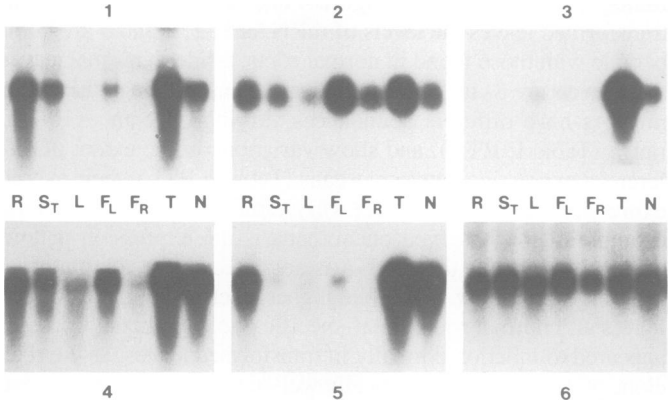

Fig. 5. Distribution of mRNAs 1–6 in normal tobacco plants grown in a greenhouse. Gel blots containing RNA from roots (**R**), stems (S_T), leaves (**L**), flowers (F_L) and green fruits (F_R) were hybridized to cDNA clones pCNT 1–6. To enable comparison of transcript levels RNAs from total SNT1005 shoots (**T**) and SNT1016 plants (**N**) were included in these experiments.

Hooft van Huijsduijnen *et al.*, 1986), indicating their involvement in plant defence mechanisms.

Extensins are abundant cell wall proteins and may be important in controlling growth and development. Our results indicate that extensin mRNA is not normally present in tobacco leaves. Therefore, the thick fleshy appearance of *T-cyt* transformed leaves and stems might be due partly to enhanced accumulation of extensins in these tissues.

Chitinase hydrolyses chitin, a major component of fungal cell walls which has not been reported to occur in plants. Therefore, it seems unlikely that chitinase has a function in normal plant growth and differentiation. Our hybridization studies demonstrate that chitinase mRNA levels are subject to tissue-specific regulation in normal axenically cultured plants. In a recent publication, similar findings were reported and negative regulation of chitinase mRNA levels by auxins and cytokinins was proposed (Shinshi *et al.*, 1987). We, however, find stimulation of chitinase mRNA levels in shoots by cytokinins. While it is conceivable that different regulatory circuits may operate in different systems, it seems premature to postulate direct regulation of chitinase mRNA levels by cytokinins alone or in conjunction with auxins. Since there is good evidence for induction of chitinase mRNA accumulation by the stress-associated phytohormone ethylene (Broglie *et al.*, 1986), regulation of pathogenesis-related genes such as chitinase by cytokinins may be mediated by ethylene (van Loon, 1983) Whether or not cytokinins cause release of ethylene might depend on additional (stress-related) physiological factors.

In spite of numerous research efforts, functions of pathogenesis-related PR-1 proteins remain unknown. The occurrence of high levels of PR-1 proteins in normal and T-DNA transformed undifferentiated callus tissues has been reported by Antoniw *et al.* (1981, 1983). Our results confirm the observation that accumulation of PR-1 gene products in uninfected tissues is correlated with (partial) inhibition of differentiation, since in normal differentiated plants PR-1 mRNA levels are very low. The levels of the PR-1-like mRNA 5 are in contrast developmentally regulated in normal plants in a similar way to extensin and chitinase mRNA levels.

In normal plants, whether cultured *in vitro* or in a greenhouse, four of the five mRNAs under study were found to exhibit similar organ-specific expression patterns. mRNA 1 is present exclusively in roots and stems, and mRNAs 2, 4 and 5 accumulate predominantly in roots to levels which are 4- to 10-fold higher than in leaves. Genes studied in other organ-specific expression systems (for a review, see Kuhlemeier *et al.*, 1987), are constitutively or inducibly expressed in certain tissues only. Stimulating factors involved in induction of non-constitutive expression in these tissues can be among others light, elicitors or phytohormones (Kuhlemeier *et al.*, 1987).

The expression of PR-1 genes seems to bear resemblance to these systems in that expression levels normally are low and can be induced by specific stimuli such as pathogenic infection, salicylic acid (Hooft van Huijsduijnen *et al.*, 1986) or cytokinins (this study). Regulation of mRNA 1, 2, 4 and 5 levels is different, as these mRNAs are normally more or less root-specific, but accumulate in shoots when root development is suppressed by cytokinins. The normal expression programmes of the corresponding genes are apparently disturbed by the high cytokinin stress.

To our knowledge comparable switches in tissue-specificity of gene expression have not been reported previously. Whether these are primarily due to cytokinin effects, or are secondary effects, for instance related to suppression of root development, remains to be determined. Experiments are under way to discriminate between these alternatives and to study the possible role of ethylene. Although the observed changes in mRNA populations may not be due to cytokinins directly, it can be concluded that the increased level of cytokinins has started a cascade of events leading to the expression characteristics described in this paper. The cDNA clones that we have isolated provide the means to study the interaction between different organs at the molecular level, and may help to elucidate the basis of tissue-specific gene expression.

Materials and methods

Plant materials and tissue culture

Origin and characteristics of *Nicotiana tabacum* var. Petit Havanna (SR1) shoots, seedlings and seed stocks used in this study have been described in detail elsewhere (Wullems *et al.*, 1981; Peerbolte, 1986; Peerbolte *et al.*, 1987). A brief description follows here. Seedlings SNT1005 and SNT1016 and seed stock were derived from sexual crosses between grafted T-DNA containing SR1-4013-3 (identical to SNT1, cf. Peerbolte *et al.*, 1987) shoots and normal SR1 tobacco plants. Upon germination on tissue culture medium, seeds give rise to two morphologically distinct types of seedlings, normal SNT1016-like and transformed SNT1005-like in a 1:1 ratio. Transformed seedlings, like their female parent SR1-4013-3, contain one copy per genome of the right part of the Octopine T_L-DNA, including

besides the cytokinin gene (*T-cyt*) genes 1, 3, 6^a and 6^b. A main root which is initially developed by transformed seedlings degenerates to callus 2–3 weeks after germination due to *T-cyt* directed overproduction of cytokinins. Upon further subculture transformed seedlings usually develop a phenotype as shown in Figure 1 for SNT1005. All tissues were grown axenically on standard hormone-free LS-medium (Linsmaier and Skoog, 1965), solidified with 0.8% agar (Difco), at 26°C and 2000 lux (12 h day). Plant stock lines were subcultured at 1 month intervals. For certain studies the medium was supplemented as indicated with 1 or 2 mg/l kinetin (Calbiochem), a synthetic cytokinin. To obtain young seedling material ~500 seeds/127-mm Petri dish were germinated on LS-medium under aseptic conditions as described (Memelink *et al.*, 1983). Two weeks after germination ~500 transformed and 500 normal seedlings were separated on the basis of their different morphology and frozen in liquid N_2. Transformed seedlings had at that time a 2.5-fold higher average fresh weight than normal seedlings. Normal tobacco Petit Havanna SR1 plants used to harvest different organs were grown for 2 months in a greenhouse.

RNA isolation

Isolation of total RNA from frozen plant tissues, and subsequent poly(A) RNA isolation by oligo(dT)–cellulose chromatography were done mainly as described by van Slogteren *et al.* (1983). A minor modification of the poly(A) RNA isolation procedure was the use of binding buffer to wash loaded oligo(dT)–cellulose columns. Unless mentioned otherwise, extraction of RNA was done 3 weeks after subculturing of plant tissues. RNA was extracted from total plantlets (To), from shoots alone (S), or from leaves (L), stems (S_T), roots (R), flowers (F_L), fruits (F_R) or callus (C). The latter tissue was usually formed at the base of SNT1005 shoots after 2–3 weeks of subculture. Total RNA yield was 200–300 μg/g fresh weight, of which 1–5% was recovered as poly(A) RNA. RNA concentrations were estimated by measuring adsorption at 260 nm, and assuming 24 A_{260} units/mg of RNA.

Construction of a cDNA library

First strand cDNA was synthesized on poly(A) RNA isolated from total SNT1005 shoots essentially as described by Maniatis *et al.* (1982). Second strand synthesis was performed subsequently in the same reaction vessel according to Gubler and Hoffman (1983) with omission of DNA ligase and β-NAD. Phosphorylated *Eco*RI linkers (P-L Biochemicals) were ligated to double-stranded (ds) cDNA according to Maniatis *et al.* (1982). Linkered ds cDNA was *Eco*RI-digested, size-selected by chromatography on Sepharose CL-4B (Pharmacia) as described by Maniatis *et al.* (1982) and cloned in the *Eco*RI site of the plasmid pUN 121 (Nilsson *et al.*, 1983). cDNA-containing transformants of *Escherichia coli* strain MH-1 were selected on carbenicillin and tetracyclin. The library consisted of 4×10^5 independent transformants, which corresponds to 10^6 transformants/μg *Eco*RI linkered ds cDNA. For long term storage, the amplified library was kept frozen in 0.7% bactopepton, 17% glycerol at −80°C.

Selection of cDNA clones

Four hundred and eighty recombinant colonies were streaked on a circular Hybond-N filter (Amersham), replicated, chloramphenicol-amplified and lysed according to Hanahan and Meselson (1980). Filters were baked, prewashed in 5 × SSPE, 5% SDS at 42°C and (pre-)hybridized and washed under conditions as described for Northern blot hybridization. Replica filters were differentially screened using radioactive (10^8 c.p.m./μg), single-stranded cDNA, synthesized on poly(A) RNA from *T-cyt* transformed SNT1005 shoots or from normal SNT1016 plants. From colonies showing differential hybridization plasmid DNAs were isolated and further screening was done by Northern blot hybridizations with the ^{32}P-labelled plasmid DNAs as probes.

Northern blot hybridization

Twenty microgrammes of total RNA were electrophoresed and blotted as described by van Slogteren *et al.* (1983). Hybridization and washing conditions were slightly modified. Baked GeneScreen (New England Nuclear) membranes were submersed for 30–60 s in 0.1 M NaOH to dissociate glyoxal–RNA complexes and neutralized in 50 mM $NaPO_4$ pH 6.5, 5 mM EDTA for at least 15 min. Prehybridizations were done in 50–100 μl/cm^2 of 5 × SSPE, 5% SDS, 50% de-ionized formamide and for hybridization 5–20 ng/ml of ^{32}P-labelled denatured probe DNA were added. Hybridized blots were washed 2–3 times in 0.1 × SSPE, 0.1% SDS heated to 65°C. To re-use blots, radioactive probes were melted off by incubation for 8 h or more in 0.1 × SSPE, 0.1% SDS, 50% de-ionized formamide at 65°C. Radioactive cDNA plasmid probes were prepared by nick translation. Transcript sizes and relative transcript abundances (RTA) were estimated using DNA fragments, generated by restriction enzyme digestion of pUN 121 vector. Known amounts of these DNA markers were glyoxylated and run in parallel with RNA samples. DNA standards were visualized on the blots by their hybridization to the vector part of radioactive cDNA plasmid probes. RNA and marker DNA hybridization signals were quantified by densitometric scanning of autoradiograms on an LKB 2202 ULTROSCAN Laser Densitometer, and the data were processed on an LKB 2220 Recording Integrator. To get an estimation of RTA in transformed leaves, mRNA 6 was arbitrarily chosen as reference mRNA and the number of molecules of each mRNA relative to mRNA 6 were calculated using the formula: $RTA_x = (I_x/I_6)\cdot(L_6/L_x)$ where I_x is the intensity of the hybridization signal of mRNA x in transformed leaf RNA relative to the DNA marker signal on the same autoradiogram and L_x is the cDNA insert length of the corresponding recombinant plasmid probe. Correction for probe specific activity was not necessary, since uniformly labelled cDNA plasmid probes were used and since mRNA hybridization signals were directly related to the DNA marker signal on the same autoradiogram. The RTA values obtained are listed in Table I.

Southern blot hybridization

Restriction enzyme-digested plasmids were electrophoresed on 1% agarose gels in Tris-borate/EDTA buffer. Gels were submersed twice in 0.25 M HCl for 15 min, once in 0.5 M NaOH for 30 min, and DNA was transferred to GeneScreen membranes using 0.5 M NaOH as transfer buffer. Membranes were neutralized for at least 15 min in 50 mM $NaPO_4$ pH 6.5, 5 mM EDTA, and baked. Hybridization and washing conditions were as described for RNA blots.

Acknowledgements

The authors thank Dr Rob Hooft van Huijsduijnen and Dr John Bol for providing plasmid DNAs of cDNAs A–F and Dr Ben Cornelissen and Dr Jan van Kan for communicating the sequencing results of pCNT 4 and pCNT 5 prior to publication. The help of Dr Bert van der Zaal in cDNA library construction and differential screening was much appreciated. Reliable technical assistance of Mr Ward de Winter in important aspects of this study and optimal greenhouse material provided by Mr Bram Wetselaar are also acknowledged.

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Received on August 14, 1987; revised on September 16, 1987