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Growth-related gene expression in *Nicotiana tabacum* mesophyll protoplasts

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Summary

Eight cDNAs whose genes are more strongly expressed in suspension cells in growth phase than in stationary phase and at a low level in mature leaves have been isolated. The corresponding mRNAs are abundantly accumulated in young plant organs and in germinating seeds but are almost undetectable in mature plant tissues and dry seeds. Six of these cDNAs were characterized by comparison of nucleotide and protein sequences to the EMBL and SWISSPROT databanks. These eight growth-related genes are expressed in protoplasts isolated from *Nicotiana tabacum* mesophyll cells shortly after preparation (4 h). Two of them are expressed in freshly isolated protoplasts (early genes), while the other six are detected after 4 h of culture (late genes). Seven are more abundantly expressed in protoplasts than in growing plant organs while one growth-related gene is weakly expressed in protoplasts, as is the histone H4 gene. They seem to be induced in protoplasts by a synergistic effect of wounding and maceration. Sustained expression of the early genes is dependent on the presence of sucrose in the culture medium.

Introduction

Higher plants present a high regeneration potential. After partial organ destruction, differentiated, non-dividing cells are able to re-enter mitosis. This cellular proliferation results in the formation of a wound callus and, under particular conditions, to root (cutting) or stem (decapitated plants) formation. This potential is particularly well expressed *in vitro* and is the basis of micropropagation and plant cell manipulation. A central feature of the totipotency of these cells is the return to growth with the reinitiation of division of non-dividing cells. Nevertheless, proliferation needs not only the expression of genes controlling the cell cycle but also genes involved in more general functions necessary for the duplication of the cell contents.

Mesophyll protoplasts are isolated from a uniform cell type and form a pipe table cell population. Under suitable conditions, protoplasts from *Nicotiana tabacum* leaves can re-enter the cell cycle and form an undifferentiated callus mass which can regenerate a whole plant identical to the original one (Nagata and Takebe, 1971). Consequently, they are an attractive model of the regeneration of plant cells. During protoplast isolation and in culture, cells undergo fundamental changes in protein synthesis (Fleck *et al.*, 1979). Most of these changes are related to a rapid modification of the mRNAs. Among genes which are affected by the dedifferentiation process, those related to photosynthesis — Rubisco (Vernet *et al.*, 1982) and a light harvesting chlorophyll *a/b* protein (Müller *et al.*, 1980) — were reported to be repressed in protoplasts, whereas others are induced, such as ubiquitin (Jamet *et al.*, 1990), glutathione peroxidase (Criqui *et al.*, 1992), glutathione S-transferase (Takahashi and Nagata, 1992), *parA* gene (Takahashi *et al.*, 1989) and the Tnt1 retrotransposon (Pouteau *et al.*, 1992). We have previously shown that mesophyll protoplasts abundantly synthesize pathogenesis-related proteins (Grosset *et al.*, 1990a) and that the corresponding mRNA accumulation is induced by the initial wounding of the leaf in the first step of protoplast isolation (Grosset *et al.*, 1990b).

The return to growth of the protoplast is of great interest because it is a crucial step for callus formation and plant regeneration. In order to understand the recovery of the growth ability of differentiated cells via protoplasts, we searched for growth-related genes and looked for their expression pattern in protoplast culture. Our approach consists of the isolation of genes which are strongly expressed during the growth phase of *N. tabacum* in suspension cells, poorly expressed during the stationary phase and not expressed in the leaf.

In this paper, we present the isolation of eight cDNAs corresponding to genes which fulfill these criteria. The nucleotide or deduced amino acid sequences of the encoded proteins of six of them present strong homologies to sequences described in the EMBL and SWISSPROT databanks. Northern blot analyses were carried out on *N. tabacum* cell suspensions, tissues and seeds, according to their developmental stage. We also studied the expression of these growth-related genes in tobacco mesophyll protoplasts and show that each corresponding mRNA is

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accumulated early in protoplast culture. Seven genes are more abundantly expressed in protoplasts than in any dividing plant tissues or organs, whereas one is expressed at a low level in protoplasts. We also attempted to define the step(s) in the isolation process which induce(s) expression of these genes.

Results

Differential screening

The cell suspension cDNA library was screened by differential colony hybridization in order to isolate clones corresponding to genes preferentially expressed during the growth process and not in differentiated leaf cells. Clones for which hybridization signals were strong with ^{32}P-labeled cDNAs from suspension cells in growth phase (3-day-old), weak with cDNAs prepared from suspension cells in stationary phase (11-day-old) and almost undetectable with cDNAs from adult leaves were selected. Plasmids were extracted from each positive clone, digested by appropriate enzymes to separate the vector from the cDNA insert, electrophoresed and transferred on to nylon membranes. Three replicas were carried out and hybridized to the same probes used for library screening. This Southern hybridization revealed that eight clones corresponded to our selection criteria.

Characterization of the eight cDNAs

In order to determine proteins encoded by the cDNAs, complete or partial sequencing was carried out and nucleotide and derived amino acid sequences were compared with the EMBL and SWISSPROT databases, respectively. The presence in two of the cDNAs of ORFs beginning with an ATG translation initiation codon indicates that they are most probably complete. The six other cDNAs have no translation initiation site and are, therefore, truncated. None of the eight nucleotide sequences has obvious polyadenylation signals upstream from the poly(A) tract. The nucleotide sequences have been submitted to the EMBL databank and are presented here under their accession number.

Accession number X58527. The complete nucleotide sequence is 699 nt long. It contains a complete ORF of 378 bp coding for a protein of 126 amino acids. The amino acid sequence shows 40–50% identity with *Chlamydomonas reinhardtii* and vertebrate thioredoxins, and only 30% with those of plant chloroplasts. In addition, the encoded protein has a perfect thioredoxin signature at position 43–49. This led us to conclude that this cDNA encodes a cytoplasmic thioredoxin. A more detailed analysis of this clone has already been presented (Marty and Meyer, 1991). Using this cDNA (thio h1) as a probe, we have recently isolated from *N. tabacum* a thioredoxin h gene (EMBL accession number Z11803) which encodes a second thioredoxin h protein (thio h2). Specific probes corresponding to these two thioredoxins were prepared (Brugidou *et al.*, 1993) and used in this study to analyze the differential expression of each gene in protoplasts.

Accession number X62500. This 937 bp cDNA shows a complete ORF of 780 nt corresponding to a protein of 260 amino acids. The nucleotide sequence has 65% identity with three eucaryotic 60S (ribosome large subunit) ribosomal protein genes. The deduced protein shows high homologies over shorter stretches with archaebacterial, eubacterial, chloroplast and mitochondrial 50S ribosomal L2 proteins, and contains the RL2 protein signature. Analyses of the nucleotide and amino acid sequences and comparison with ribosomal L2 proteins were described in a separate publication (Marty and Meyer, 1992).

Accession number Z14085. The 389 bp cDNA contains a partial ORF of 231 bp corresponding to 77 amino acids. The 3′ untranslated region is 140 bp and is followed by a 21 nt poly(A) tract. The partial nucleotide sequence was compared with the EMBL database and presents high homology with two eucaryotic sequences (60.8% identity over 237 nt with the first, coding for a *Trypanosoma brucei* hypothetical protein U (Vijayasarathy *et al.*, 1990), and 63.2% identity over 155 nt with the second, coding for a *Dictyostelium discoideum* ribosomal protein named 1024 (Steel *et al.*, 1987)). The partial protein sequence of 77 amino acids shows high homology with these two deduced eucaryotic proteins (Figure 1). In addition, good homologies are detected with procaryotic and chloroplastic ribosomal S4 proteins (RS4) over a short stretch of approximately 25 amino acids which correspond to a highly conserved region of the S4 proteins (Figure 1). A prosite-specific signature for the ribosomal S4 proteins has been established in the conserved region [AS]R[QV]L[IV]X[QHG]X[HA]IX[VI]. The two eucaryotic protein sequences — *T. brucei* hypothetical protein U and *D. discoideum* ribosomal protein — and our tobacco partial sequence, but no other eucaryotic protein, match this RS4 signature pattern (Figure 1). These results suggest that the hypothetical protein of *T. brucei* and the ribosomal protein 1024 of *D. discoideum* are probably eucaryotic ribosomal S4 proteins. The much greater homology of the nucleotide and protein sequences of our clone with the eucaryotic rather than procaryotic ribosomal proteins suggests that this cDNA encodes a tobacco cytoplasmic 40S ribosomal S4 protein.

Accession numbers Z14079 and Z14080. The sequencing of the 5′ and 3′ extremities of a 700 bp cDNA was carried out. The 5′ sequence of 156 bp presents a potential

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                 120        130        140                  150        160
TOBCY      AKSVHHARVLIRQRHIRVGRQVVNVPSFMVRL--------DSQKHIDFSLIS
TRYBR      ......S....Q....A.AK.I.TI...I..V--------S.EH..A.ADA.
DICDI      ...I.......KG......K.L......L..V--------E....LGLAST.
ORYSA      -STIPE..Q.VNH...L.NGRI.DI...RCKPRDIITTK.N.RSKRLVQN.
MAIZE      .STIPG..Q.VNH...L.NGRI.DI...RCKPRDIITTK.N.RSKRLVQNY
SPIOL      .PTIPG..Q.VNH...L.NGRI.DI..YRCKPQDTIMAR.E..S.ALIQN.
TOBAC      .STIPA..Q.VNH...L.NGRI.DI..YRCKPRDIITAK.E..SRALIQ..
MARPO      .PTIPG..Q.VNH...LINNNT.DI..YNCKPKDVITIK.RS.SQSIIIKN
BACSU      .RTRRQ..Q.VNHG..L.DGSR.DI..YL.KPGQTIGVREKSRNLSIIKE.
ECOLI      GATRAE..Q.VSHKA.M.NGR...IA.YQ.SPNDVVSIREKA.KQSRVKAA
CRYPH      -PTIPA..Q.VNHG..K.NNTR.SI..YQCKAGDMISIRQHP.SQSIVKNY
consens      .   .* *.    * .    * ..*.
                   110        120        130        140        150

                  170        180             190
TOBCY      PFGGGRPGRVKRKNQKAAAKKASGGDGDEEDEE
TRYBR      ...N........VK-RN....G...G-DDDE
DICDI      .LA......RA..MA.NNSS.G-------.E-.N
ORYSA      IASSDPGKLP.HLTIDTLQY.GLVKKILDRKWVGLKINELLVVEYYSRQT
MAIZE      IASSDPGKLP.HLTVDTLQY.GLVKKILDRKWVGLKINELLVVEYYSRQT
SPIOL      LDLSP.EELP.HLTLNPFPY.GLVNQIIDSKWVGLKINELLVVEYYSRQT
TOBAC      LDSSPHEELPNHLTLHPFQY.GLVNQIIDSKWVGLKINELLVVEYYSRQT
MARPO      LNSFQKQKIPNHLTFDLMQI.GLVNQIIDREWIYLKINELLVVEYYSRQV
BACSU      VEVNNFV--PEYLTFD.EKLEGTFTRLP.RSELAPEINEALIVEFYSR--
ECOLI      LELAEQREKPTWLEVD.GKMEGTFKRKP.RSDLSADINEHLIVELYSK--
CRYPH      LQFP.LANMPNHLQIDKDNLTGKINGII.R.WVAL--NELLIVEYYSRKG
consens                          .        .
                  160        170        180             190        200
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Figure 1. Partial alignment of RS4 protein sequences.
(.) Corresponds to identical and (–) to gaps introduced to optimize alignment. In consensus sequence, (*) indicates amino acids identical in all sequences and (.) amino acids with similar biochemical characteristics. Upper amino acid labeling is done according to *T. brucei* and lower to *Cryptomonas phi* sequences. TOBCY: *N. tabacum* cytoplasmic ribosomal protein S4; TRYBR: *T. brucei* hypothetical protein U; DICDI: *D. discoideum* ribosomal protein 1024; ORYSA: *Oryza sativa* chloroplastic ribosomal protein S4; MAIZE: maize chloroplastic RS4; SPIOL: *Spinacia oleracea* chloroplastic RS4; TOBAC: *N. tabacum* chloroplastic RS4; MARPO: *Marchantia polymorpha* chloroplastic RS4; BACSU: *Bacillus subtilis* RS4; ECOLI: *Escherichia coli* RS4; CRYPH: *Cryptomonas phi* RS4.

coding region of 52 amino acids, the 3′ sequence of 356 bp one of 51 nucleotides encoding 17 amino acids followed by 289 bp of untranslated sequence and a 16 nt poly(A) tract. These partial nucleotide sequences show very high homology with the translation elongation factor-1α (EF-1α) gene of tomato (Shewmaker *et al.*, 1990) (92.4% identity over 157 bp for the 5′ region and 73.9% identity over 357 bp for the 3′ region) and the EF-1α A4 gene of *Arabidopsis thaliana* (Liboz *et al.*, 1989, 84.5% identity over 129 bp for the 5′ region and 58% identity over 274 bp for the 3′ region). In addition, these nucleotide sequences give good homologies with other eucaryotic and procaryotic EF-1α sequences. The deduced amino acid also shows very good homology with tomato EF-1α: 88.5% identity over 52 amino acids for the 5′ extremity and 100% over 17 amino acids from the 3′ region (Figure 2) and with all other eucaryotic EF-1α sequences. These very high homologies of the nucleotide sequence and the corresponding partial protein to EF-1α allow us to define this partial cDNA sequence as a tobacco EF-1α cDNA.

Accession numbers Z14081 and Z14082. An approximately 700 bp long cDNA was sequenced from both ends. The 5′ sequence is 168 nt long, encoding 56 amino acids. The 3′ sequence is 210 nt long followed by a 33 nt poly(A) tract. These nucleotide sequences present high homology with eucaryotic and procaryotic sequences encoding cyclophilin, CyP. The best homology was found with the tomato CyP nucleotide sequence (Gasser *et al.*, 1990): 89.2% identity over 167 nt for the 5′ region and 71.1% identity over 190 nt in the 3′ region. In addition, the peptide deduced from the 5′ extremity shows the best homology with the tomato CyP protein – 98.3% identity over 56 amino acids (Figure 3). It also presents high homology with all CyP sequences described in the literature. These very high homologies of the nucleotide and corresponding amino acid sequences with CyP confirm that this cDNA encodes a tobacco CyP.

Accession number Z14084. The sequence of 620 nt was established, in which 450 nt encode a partial ORF of 150

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                330         340         350         360         370         380
EF1TOBACO       TVQVIIMNDTGQIGNGYAPVLDCHTSTLTVKFAEILTKIDRRSGKELEKEPK_________
EF1TOMATO       TAQVIIMNHPGQIGNGYAPVLDCHTSHIAVKFAEILTKIDRRSGKELEKEPKFLKNGDAG
EF1ARABTH       TSQVIIMNHPGQIGNGYAPVLDCHTSHIAVKFSEILTKIDRRSGKEIEKEPKFLKNGDAG
EF1EUGLGR       TAQVIILNHPGQIGNGYAPVLDCHTCHIACKFATIQTKIDRRSGKELEAEPKFIKSGDAA
consens         * ****.*. ************ ***  .. **. * **********.* ***

                390         400         410         420         430         440         450
EF1TOBACO       ____________________________________________________KKDPTGAKVTKAAQKK--K
EF1TOMATO       MVKMIPTKPMVVETFAEYPPLGRFAVRDMRQTVAVGVVKNVDKKDPTGAKVTKAAQKKG-K
EF1ARABTH       MVKMTPTKPMVVETFSEYPPLGRFAVRDMRQTVAVGVIKSVDKKDPTGAKVTKAAVKKGAK
EF1EUGLGR       IVLMKPQKPMCVESFTDYPPLG-VSCGDMRQTVAVGVIKSVNKKENTG-KVTKAAQKK--K
consens                                                     **. ** ****** **  *
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Figure 2. Partial alignment of plant translation elongation factor-1α sequences.
(–) Corresponds to gaps introduced to optimize alignment, (_) shows the non-sequenced part of the tobacco EF-1α. In consensus sequence, (*) indicates amino acids identical in all sequences and (.) indicates amino acids with similar biochemical characteristics. Amino acid labeling was done according to tomato EF-1α. (EF1TOBACO: EF-1α of *N. tabacum*, EF1TOMATO: EF-1α of *Lycopersicon esculentum*, EF-1ARABTH: EF-1α of *Arabidopsis thaliana*, EF-1 EUGLGR: EF-1α of *Euglena gracilis*).

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                102         110         120         130         140         150
CYPTOBAC            PGFLSMANAGPGTNGSQFFICTAKTEWLNGKHVVFGQVVEGYDVIKKAE
CYPTOMAT        PGILSMANAGPGTNGSQFFICTAKTEWLNGKHVVFGQVVEGMDVIKKAE
CYPMAIZE        PGVLSMANAGPNTNGSQFFICTVATPWLDGKHVVFGQVVEGMDVVKAIE
CYPHUMAN        PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEGMNIVEAME
CYPMOUSE        PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEGMNIVEAME
CYPRAT          PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEGMSIVEAME
CYPBOVIN        PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGKVKEGMNIVEAME
CYPSCHPO        PGLLSMANAGPNTNGSQFFITTVVTPWLDGKHVVFGEVTEGMDVVKKVE
consens         ** ******** ******** *  **** ******* * **  ..   *

CYPTOBAC        AVGSGSG
CYPTOMAT        AVGSSSG
CYPMAIZE        KVGTRNG
CYPHUMAN        RFGSRNG
CYPMOUSE        RFGSRNG
CYPRAT          RFGSRNG
CYPBOVIN        RFGSRNG
CYPYEAST        SLGSPSG
consens           *. .*
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Figure 3. Alignment of partial protein sequences of cyclophilin homologs.
(–) Correspond to gaps introduced to optimize alignment. In consensus sequence, (*) indicates amino acids identical in all sequences and (.) indicates amino acids with similar biochemical characteristics. Amino acid labeling was done according to tomato CyP. (CYPTOBAC: *N. tabacum* cyclophilin; CYPTOMAT: *L. esculentum* cyclophilin; CYPMAIZE: *Zea mays* cyclophilin; CYPHUMAN: *Homo sapiens* cyclophilin; CYPMOUSE: *Mus musculus* cyclophilin; CYPRAT: *Rattus norvegicus* cyclophilin; CYPBOVIN: *Bos taurus* cyclophilin; CYPSCHPO: *Schizosaccharomyces pombe* cyclophilin).

amino acids. The 3′ noncoding region is 170 nt long followed by a 10 nt poly(A) tract. The nucleotide and deduced amino acid sequences show high identity with human (Adams *et al.*, 1991; Dowdy *et al.*, 1991; EMBL accession number M73791), mouse (EMBL accession number X61826) and rice sequences (EMBL accession number X64621) (Rivera-Madrid *et al.*, 1993). The human cDNAs, isolated from kidney (Dowdy *et al.*, 1991), mammary gland and brain (Adams *et al.*, 1991), are identical. Some of these sequences were identified by systematic sequencing (mouse, rice and two human sequences) and nothing is known as to the role played by the corresponding proteins. Only the Wilms' tumor kidney cDNA has been characterized for its ability to suppress the tumoral phenotype. Consequently, we use the terminology of tumor-related protein.

Clones GA and GB. The sequencing of the 5′ and 3′ extremities of two other cDNAs (700 and 1500 bp) was

carried out. The nucleotide sequences and the potential ORFs of each one do not reveal obvious homologies with known sequences in the EMBL, GenBank, SWISSPROT and GenPept databases.

Expression of the corresponding genes in different organs and tissues of tobacco

Expression in cell suspensions and expanded leaves. According to the differential screening, the steady-state level of each mRNA is higher in cell suspension during the exponential phase than during the stationary phase, and at a low level in expanded leaves. The levels of expression of each gene were analyzed by Northern hybridization: 10 μg of total RNA from growth and stationary phase cell suspension and from expanded leaves were electrophoresed and transferred on to membranes. The corresponding Northern blot was first hybridized with an *Arabidopsis* histone H4 probe as a control to indicate tissues in division, and then with each cDNA probe (Figure 4a). As expected, histone H4 mRNA is more abundant in cell suspension in growth phase than in stationary phase

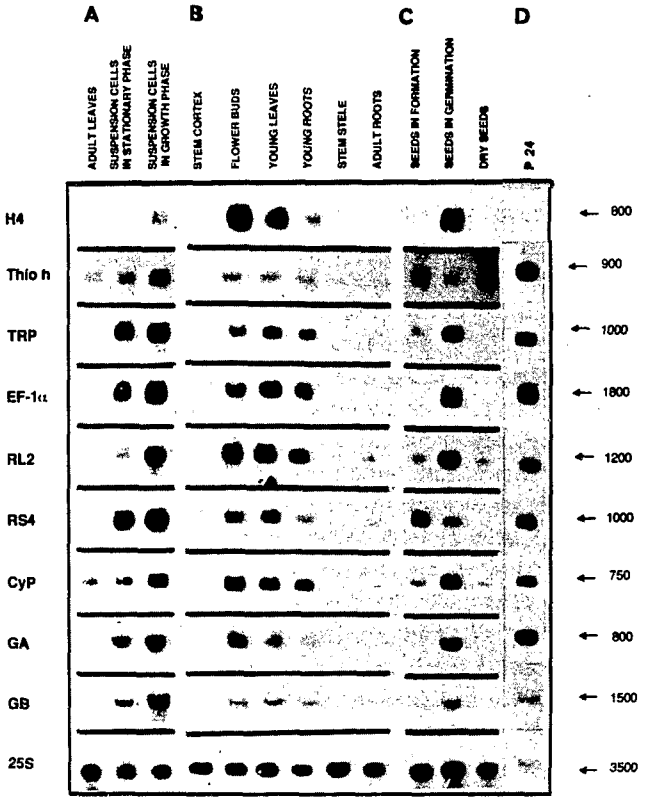

Figure 4. Accumulation of mRNAs homologous to the eight cDNAs and to histone H4 in *in vitro* cultivated cells (A), in different tissues (B), organs (C) and 24-h-old protoplasts (D). Ten micrograms (A, B, C) or 2 μg (C) of total RNAs extracted from each sample were electrophoresed and blotted on to nylon membranes. Thio: thioredoxin; TRP: tumor-related protein; EF-1α: translational elongation factor-1α; L2 and S4: cytoplasmic ribosomal proteins L2 and S4; CyP: cyclophilin; GA: gene A; GB: gene B; H4: *Arabidopsis* histone H4.

and almost undetectable in expanded leaves. With different intensities of hybridization signals, each gene shows an expression pattern more or less comparable with that of histone H4. However, the EF-1α, RS4, TRP and GA mRNAs are also largely accumulated in cell suspension in stationary phase, although significantly less than in the growth phase, and weakly in expanded leaves. The mRNA sizes are compatible with those of the putative proteins (given in Figure 4) confirming the characterization of these cDNAs.

Expression in vegetative tissues. Total RNA was extracted from tissues of expanded (3-month-old) and young (2-week-old) tobacco plants. Tissues were chosen according to their developmental state: young ones in which cells are dividing such as young leaves, young roots and flower buds, and mature ones with no cell division such as adult roots, stem stele and stem cortex. After electrophoresis and transfer of 10 μg of total RNA from each tissue, the *Arabidopsis* H4 histone and the seven cDNA probes were hybridized to the corresponding Northern blot (Figure 4b). The H4 histone transcripts are abundantly accumulated in young tissues in which cell division is known to occur and are almost undetectable in expanded ones. Expression of each gene is very high in young tissues – young leaves, young roots and flower buds. In contrast, each mRNA was detected at a low level in mature tissues – stem cortex and stele, adult roots, adult leaves (as previously shown). These results, compared with the expression pattern of the *Arabidopsis* H4 histone gene, indicate that the eight genes identified in this study show a specific expression in growing tissues.

Expression in reproductive tissues. Total RNA was extracted from tobacco seeds during late formation (storage protein accumulation and desiccation, no cell division), from dry seeds and from 2-day-old germinating seeds (active cell divisions). After electrophoresis and transfer of 10 μg of each RNA, the *Arabidopsis* H4 histone and the eight cDNA probes were hybridized to this Northern blot (Figure 4c). The H4 histone mRNA is strongly accumulated in germinating seeds and is detected at a very low level during formation and in dry seeds. Five genes follow the same pattern of expression as histone H4. The RS4 mRNA is not only strongly accumulated in germinating seeds but is also detectable at a high level in seeds in formation. The thioredoxin gene presents a different expression pattern compared with the others. It is strongly expressed in seeds in formation, less in dry seeds and at a very low level in germinating seeds.

Expression of the corresponding genes in protoplasts

Growth-related genes are overexpressed in protoplasts. The expression of these growth-related genes was

analyzed in tobacco mesophyll protoplasts which, under suitable conditions, can re-enter the growth phase and divide. Gene expression was tested in 24-h-old protoplasts which are at the end of the G1 phase of the cell cycle (Meyer and Cooke, 1979; Zelcer and Galun, 1980). The expression pattern of these genes was compared with that of histone H4, which is strongly expressed in proliferating cells. Total RNA (2 μg) from 24-h-old protoplasts and 10 μg from growing tissues (young leaves and suspension cells in exponential phase) and mature cells (expanded leaves) were run on the same gel. The RNA was transferred on to nylon membranes and hybridized with each growth-related cDNA, histone H4 and rDNA probes (Figure 4d). As previously shown, each mRNA is more abundantly accumulated in growing tissues than in mature ones. In addition, each growth-related gene is expressed in protoplasts at 24 h of culture. The comparison of the hybridization signals between protoplasts (2 μg of total RNA per lane) and tissues (10 μg of total RNA per lane) shows that seven growth-related genes are more strongly expressed in protoplasts than in growing tissues. Compared with cell suspension during the growth phase, mRNa levels are approximately 10 times higher for thioredoxin h and GA and four times higher for TRP, EF-1α, RL2, RS4, and CyP. In contrast, the accumulation of GB and H4 histone mRNAs is almost undetectable in 24-h-old protoplasts.

Early expression in protoplast culture. The time course of mRNA accumulation for each gene was followed after protoplast isolation (0 h) or after 4, 8, 14, 24 and 48 h of culture. Total RNA (2 μg) from each time point was electrophoresed and transferred on to a nylon membrane. Each cDNA probe was hybridized to the corresponding Northern blots (Figure 5). Thioredoxin h and tumor-related protein genes are already abundantly expressed in freshly isolated protoplasts. In contrast, the two cytoplasmic ribosomal proteins, translation elongation factor-1α, cyclophilin, GA and GB genes are expressed after 4 h of culture. The accumulation of each mRNA increases during protoplast culture, at least until 48 h. This differential expression in protoplast culture allows us to distinguish two groups of genes – group I in which genes are expressed in freshly isolated protoplasts and group II in which genes are expressed at least 4 h after culture. As the GB gene is only weakly expressed during protoplast culture, it was not studied further.

Inducing steps during protoplast isolation. As the cDNAs correspond to genes expressed early in protoplast culture, we looked for the steps of protoplast isolation which may trigger their expression. The protoplast preparation procedure consists of a series of treatments applied in the following chronological order: leaf harvesting, sterilization, strip cutting, cell plasmolysis, infiltration under vacuum,

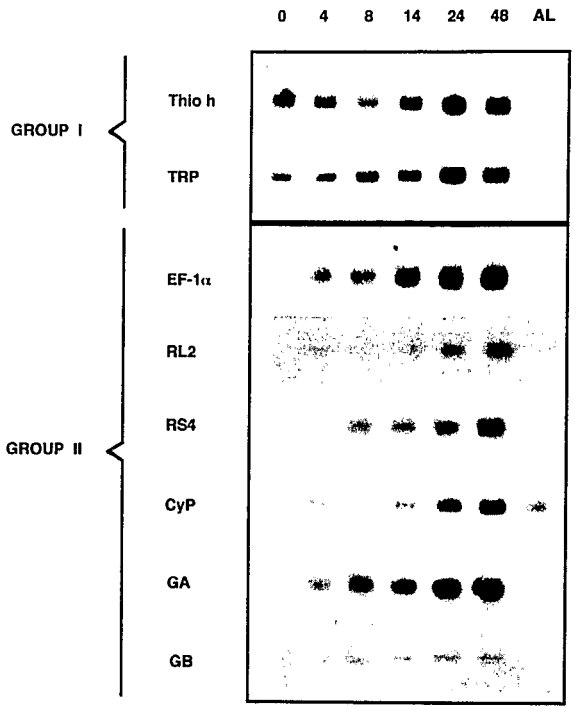

Figure 5. Time course of accumulation of mRNAs homologous to the eight growth-related and histone H4 clones during the culture of tobacco mesophyll protoplasts.
Two micrograms of total RNA extracted from freshly isolated protoplasts (0 h) or after 4, 8, 14, 24 and 48 h of culture, and 10 μg from adult leaves were electrophoresed and blotted. The same abbreviations of genes are used as in Figure 4.

wall digestion by pectinase and cellulase enzymes, centrifugation to collect and wash protoplasts, and culture in the medium (W0.6). The effect of each step was tested after 18 h of culture in order to compare it with protoplast culture at 14 h since the preparation of protoplasts takes 4 h. Expression of each gene was compared with the basal expression in adult leaves (Figure 6, first lane). To analyze the effect of the first steps, sterilized leaf discs (8 cm diameter) were cultivated 18 h in W0.6, the edges (5 mm wide) of these discs were removed and the central parts were collected for total RNA extraction. A weak expression of the eight genes was found after this treatment (Figure 6, second lane). To study the consequences of strip cutting on gene expression, leaf strips (1 mm wide) were placed on W0.6 and collected after 18 h of culture. The seven mRNAs accumulate at levels approximately five times lower than in protoplasts (Figure 6, third lane). The effect of cell plasmolysis on gene expression was tested by placing leaf strips for 18 h on twofold diluted Murashige and Skoog medium, which has a low osmotic pressure but the same nutritional and hormonal elements as W0.6 (Figure 6, fourth lane). The expression of these genes in leaf strips cultivated in MS and W0.6 is similar. Infiltration under vacuum has no significant influence on the expression pattern of the eight genes (data not shown). However, the

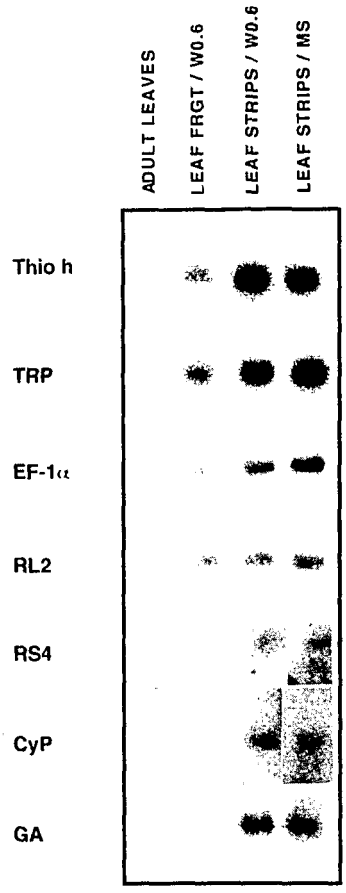

Figure 6. Detection of mRNAs homologous to seven growth-related clones during protoplast isolation.
Ten micrograms of total RNA extracted from adult leaves, large leaf fragments or leaf strips cultivated for 18 h on W0.6 or MS media, were electrophoresed and blotted. The same abbreviations of genes are used as in Figure 4.

culture medium could possibly act as a synergistic factor in their induction. Thus, we looked for the effect of major elements of the culture medium on the induction of gene expression, both in leaf strips and protoplasts.

Effect of the culture medium on gene expression. No expression of the seven genes was found when leaf strips were placed on water, showing a clear effect of the culture medium. The effects of several major nutritional elements (sucrose, iron, macroelements, microelements and vitamins) were analyzed. These experiments were carried out for the seven genes abundantly expressed in protoplasts. As the results are similar within each group, we only show results for thioredoxin h for group I and elongation factor-1α for group II. Expression of group I genes is dependent on sucrose in the culture medium, while expression of group II genes does not appear to be modified by omitting one group of the nutritional components of W0.6. This shows that different groups of nutritional elements composing the culture medium are exchangeable in inducing the

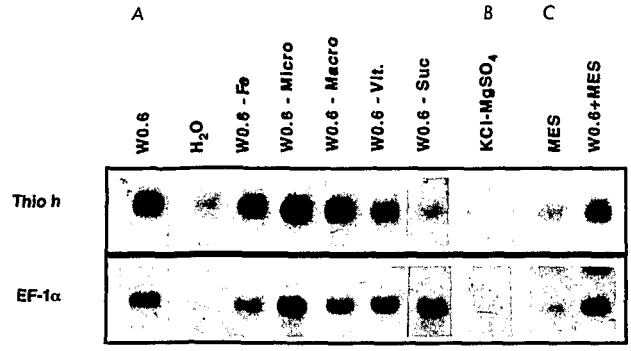

Figure 7. Effects of W0.6 culture medium elements on the accumulation of mRNAs hybridizable to thioredoxin h (Thio) and elongation factor-1α (EF-1α) cDNA probes.
Leaf strips were cultivated for 14 h (A) in water, in complete W0.6 culture medium or in culture medium lacking one nutritional component (iron, microelements, macroelements, vitamins or sucrose), (B) in KCl–$MgSO_4$ and (C) in MES buffer and in MES plus W0.6 culture medium.

expression of group II genes, suggesting an effect on a general parameter, for example, the pH control. The effect of the buffer MES (2-[*N*-Morpholino] ethane sulfonic acid) (pH 5.5) was analyzed by placing leaf strips on MES dissolved in water or in W0.6 (Figure 7c). Although MES is not toxic, it is not able induce the expression of group II genes.

We looked for effects of phytohormones on the induction of growth-related genes since auxin is required for division of protoplasts (Nagata and Takebe, 1971). Expression of each gene was tested by cultivating protoplasts in W0.6 medium with, or without, auxin and collecting samples from each culture at 4, 8 and 14 h. Total RNA (2 μg) from each sample was electrophoresed and transferred on to nylon membranes. The cDNA probes were hybridized to corresponding Northern blots (Figure 8). Cyclophilin and ribosomal protein L2 mRNAs are accumulated at a slightly lower level in the presence of this hormone. Only the level of the mRNA encoding thioredoxin h is clearly modulated by auxin: freshly isolated protoplasts present a high level of thioredoxin h mRNA which remains almost unchanged during the culture in the complete medium. In the absence of auxin the mRNA level first drops and then increases. This expression pattern has been observed reproducibly on Northern blots which have hybridized with the other probes first, thus eliminating the possibility of an artifact. We have isolated two *N. tabacum* thioredoxin h clones (thio h1 and thio h2, Brugidou *et al.*, 1993). When the membrane is hybridized with the probe specific to thio h1, it appears that thio h1 mRNA is present in freshly isolated protoplasts and that the level is slightly reduced during culture in the presence of auxin and to a greater extent in the absence of this hormone. Hybridization of the membrane with the h2 specific probe shows that thioredoxin h2 mRNA is not detectable in freshly isolated protoplasts but only later and at a low level in culture. In addition, thioredoxin

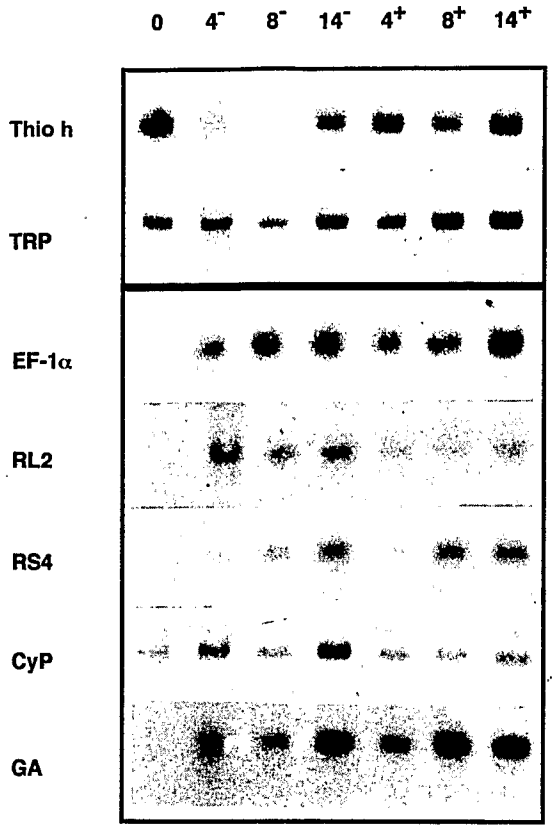

Figure 8. Time course of accumulation of mRNAs homologous to seven growth-related clones in protoplasts cultivated in media with, or without, auxin.
Two micrograms of total RNA extracted from freshly isolated protoplasts and from protoplasts cultivated 4, 8 and 14 h were electrophoresed and blotted. The same abbreviations of genes are used as in Figure 4.

h2 mRNA is more abundant without auxin. Based on the kinetics the thioredoxin h1 gene belongs to group I (early expressed gene), and the thioredoxin h2 gene belongs to group II (late genes). Nevertheless, the levels of thioredoxins h1 and h2 mRNAs do not completely explain the observed expression pattern of mRNAs hybridizable with the entire thioredoxin h1 probe. A reassessment of the genes encoding thioredoxins h is needed to clarify this point.

Finally we tested the effect of prolonged maceration. A clear difference between early and late genes is that the mRNAs of the former are detectable during maceration, while those of the latter are only detectable during culture using our standard isolation protocol (1.5 h maceration with Pectin Acid Trans Eliminase followed by 1.5 h with a cellulase–pectinase mixture). In order to determine if the transfer into the culture medium plays a role in the induction of these genes, we macerated leaf explants overnight in the cellulase mixture. All seven mRNAs are detectable in protoplasts at the end of this maceration, roughly at the same level as when they are transferred into the culture medium and cultivated overnight, indicating that the maceration medium is compatible with the induction of the late genes.

Discussion

Expression of growth-related mRNAs and function of the encoded proteins

The first goal of our study was the isolation of cDNA clones corresponding to genes whose expression is related to proliferation. Clearly the eight clones complementary to mRNAs isolated from a cell suspension fulfill this requirement: their mRNAs are detectable in all young plant tissues but not in mature tissues. Nevertheless, they are not regulated in exactly the same way, as shown by Northern blots. In addition, the characterization of the proteins encoded by six of them suggests that they are implicated in different cellular processes.

Ribosomal components. Three cDNAs encode proteins which are components of the ribosome, i.e. are involved in the same process of protein biosynthesis. Hybridization with these probes clearly indicates that ribosome synthesis is associated with proliferation and is not detectable in non-dividing tissues, even those which show very active protein synthesis (Marty and Meyer, 1992). Similar results were found in maize by Lebrun and Freyssinet (1991) who observed a greater accumulation of maize cytoplasmic S11 ribosomal protein transcripts in growing tissues than in expanded leaves and for the elongation factor-1α genes in *Arabidopsis* and tomato (Liboz, 1990; Pokalsky *et al.*, 1989; Ursin *et al.*, 1991). Nevertheless, Northern blots indicate that mRNAs encoding ribosomal proteins RL2 and RS4 are not always present in the same proportions in different tissues, although the proteins have to be in stoichiometric amounts. It is possible that they are under different translational control. This observation could also be due to the hybrid origin of *N. tabacum*: one or both probes may hybridize preferentially to mRNAs derived from one subgenome and each subgenome may participate differently in ribosome synthesis in different tissues. More generally, it is possible that these genes, RL2, RS4 and EF-1α, belong to a multigenic family and that, in our experiment, we detect genes of each family which are not regulated in the same manner. This is supported by the fact that in *A. thaliana*, the four genes which encode EF-1α are differently regulated (Liboz *et al.*, 1989).

Post-translational modificating enzymes. Thioredoxin reduces specific disulfide bonds in targeted proteins (Homlgren, 1985). In higher plants and green algae, two thioredoxin systems have been characterized: the first is localized in the chloroplasts and plays a key role in photosynthesis in regulating enzymes of CO_2 fixation

(Decottignies *et al.*, 1990, 1991; Van Langendonckt and Vanden Driessche, 1992). Corresponding cDNA clones have been isolated and sequenced (Kamo *et al.*, 1989; Maeda *et al.*, 1986; Wedel *et al.*, 1992). The second thioredoxin activities (h type) are present in heterotrophic cells (Johnson *et al.*, 1987a, 1987b; Vogt and Follmann, 1986) and are most probably localized in the cytoplasm (Florencio *et al.*, 1988; Marcus *et al.*, 1991). Recently we have isolated a cDNA (Marty and Meyer, 1991) and a genomic clone (Brugidou *et al.*, 1993) which encode thioredoxins h. Both share structural similarities with animal and yeast thioredoxins which have been shown to be associated with proliferation (Bhat *et al.*, 1989; Flamigni *et al.*, 1989; Grippo *et al.*, 1985; Müller, 1991; Reichard, 1988; Wiegand *et al.*, 1989). Our results show that thioredoxin h genes are expressed in growing cell suspension and plant tissues, but their expression is more abundant during seed formation and in dry seeds than during germination. These results are in agreement with the presence of thioredoxin activity in these same tissues (Betersmann *et al.*, 1981; Kobrehel *et al.*, 1991).

Cyclophilin (CyP) is a ubiquitious protein first discovered as the target of cyclosporin A, an immunosuppressor able to block tissue rejection by inhibiting the proliferation of T lymphocytes (Jenkins *et al.*, 1988). Recently, CyP has been shown to be identical to a previously described enzyme, peptidyl-prolyl *cis–trans* isomerase (rotamase) that catalyzes the *cis–trans* isomerization of proline-containing oligopeptides, and which accelerates the folding of several proteins (Fischer *et al.*, 1989). In addition, cyclophilin interacts with calcineurin, a Ca^{2+}-dependent phosphatase (Schreiber, 1992). In humans, three CyP isoforms have been identified (Bergsma *et al.*, 1991). In tomato, a high level of CyP mRNA was found in young leaves, floral buds, growing shoots and stamens at anthesis (Gasser *et al.*, 1990) as well as in stressed tissues (Marivet *et al.*, 1992). Our results confirm those concerning the strong expression of the CyP gene in young tissues. The CyP gene is also expressed in mature tissues but significantly less than in young ones.

Tumor-related protein. One clone shows high homology to cDNA sequences isolated from human tissues – Wilms' kidney cells (Dowdy *et al.*, 1991), mammary gland and brain – mouse preadipocytes and rice seed aleurone. We have recently isolated a full-length cDNA clone from *A. thaliana* and shown that the high homology between the human and plant-encoded proteins extends over the entire molecule (Rivera-Madrid *et al.*, 1993). Studies on the function of this gene have only been carried out on Wilms' cells (kidney). The mRNA is not detected in tumor cells (Dowdy *et al.*, 1991). Transfection of this gene into a Wilms' tumor cell line reverted the tumorigenic phenotype. Thus, this gene could be associated with some mechanisms limiting cell differentiation in humans. This mRNA sequence has been shown to be co-expressed with a laminin receptor gene in different tumor and normal human tissues (Bignon *et al.*, 1992). The Wilms' tumor proteins contain a potential nuclear targeting signal but no other obvious function can be deduced presently from its sequence. The presence of homologous cDNA sequences in vertebrates and plants and the high conservation of the encoded protein suggest a general role in eucaryotic cell metabolism.

Expression of growth-related genes in protoplasts

From mesophyll to suspension cell. The most salient result is the very early expression of the eight marker genes after protoplast isolation. As previously described (Fleck *et al.*, 1979), the transition from a mesophyll cell to a protoplast triggers rapid changes in protein synthesis. Our results show that eight randomly selected genes related to growth in the cell suspension are turned on very early in protoplasts. Apart from GB, which is expressed at a low level in protoplasts, the seven other clones are expressed at a higher level than in suspension cells, using total RNA as an internal standard, i.e. a reference which is fairly constant in mesophyll cells during protoplastization and early culture (Cooke and Meyer, 1981). The high expression of genes implicated in protein biosynthesis, such as ribosomal proteins and translation elongation factor-1α, suggests an increase in capacity of the translational system necessary for active protein biosynthesis. It is possible that thioredoxin h1, h2, and cyclophilin are required at a higher level in protoplasts for the correct folding of some newly synthesized proteins. The differential expression of thioredoxins h1 and h2 in protoplast culture suggests that the corresponding proteins have different targets. Most of the genes described as newly and early expressed in mesophyll protoplasts have also been shown to be expressed in callus or cell suspensions, including those encoding basic pathogenesis-related proteins (Grosset *et al.*, 1990), ubiquitin (Jamet *et al.*, 1990), CEP (Genschick *et al.*, 1990), HMG-CoA-reductase and glutathione peroxidase (Criqui *et al.*, 1993): from the point of view of gene expression a protoplast becomes a callus cell just after protoplast isolation or even during maceration if an overnight method is used.

The expression of the growth-related genes is not dependent on cell cycling. Re-entry into proliferation involves cell cycling but also general mechanisms such as synthesis of membranes, cell walls and organelles necessary for cell doubling. In tobacco protoplasts, progression through the cell cycle takes place only in the presence of auxin and cytokinin in the culture medium (Meyer and Chartier, 1981). In the tissues of the entire plant the expression of the growth-related genes is relatively well correlated with that

of the histone H4 gene, a gene known to be expressed just before, and during, the S phase of the cell cycle. In protoplasts the eight mRNAs are detectable independently of the S phase which occurs after 30 h in culture in complete medium (Cooke and Meyer, 1981; Meyer and Cooke, 1979). In contrast, histone H4 mRNA is almost undetectable during the early phase of protoplast culture and remains at a low level in comparison with suspension cells. This is probably related to the duration of the first cell cycle of mesophyll protoplasts: the faster protoplasts divide after 2 days but it is only after 6 days in culture that 80% of the protoplasts have undergone mitosis (Zelcer and Galun, 1980), while suspension cells in growth phase cycle in about 15–20 h. The low abundancy in protoplasts of cycle-dependent mRNAs, which are a subset of auxin-dependent mRNAs, has been directly shown by Takahashi *et al.* (1989) and Takahashi and Nagata (1992): out of 15 000 clones from a protoplast cDNA library, only two genes, *parA* and glutathione S-transferase, are induced by auxin. Nevertheless, the absence of cycle control on the expression of a gene does not exclude that the encoded protein plays a role in cell cycle progression.

Isolation step and signal inducing the expression of the growth-related genes. The expression of these genes can be induced in leaves by cutting them into strips. Plasmolysis has no additional effect. In contrast, maceration increases the mRNA levels five times if we compare 1 mm long strips with protoplasts. The fact that the relative levels of the eight mRNAs are the same in strips and protoplasts suggests that the signals released by wounding and maceration may be identical. In addition, the signal released by wounding appears to be unable to migrate into the leaf tissue (low induction in large explants) even using vacuum infiltration of the culture medium. The simplest hypothesis to explain these observations is that only a few cells are induced in leaf strips near the excision sites, while most protoplasts are induced. Other genes which are abundantly expressed in protoplasts such as ubiquitin (Jamet *et al.*, 1990), glutathione peroxidase (Criqui *et al.*, 1993) and HMG-CoA-reductase (Criqui, 1992) are also expressed in wounded leaves.

The growth-related genes are not induced in leaf strips maintained in water or in protoplasts cultivated in KCl+$MgSO_4$, while they are induced in the maceration medium which uses the same osmotic stabilizer and hydrolytic enzymes. The maceration probably releases sugars which fulfill the sucrose dependency of the early genes. The late genes are dependent on another factor common to the maceration medium and the culture medium which has not been characterized at the present time.

In previous work (Grosset *et al.*, 1990a, 1990b) we have shown that protoplasts synthesize basic, vacuolar pathogenesis-related proteins and that the accumulation of the corresponding mRNAs results from the wounding of the leaf in the early step of the isolation procedure. This conclusion is also true for the accumulation of the mRNAs of the growth-related genes, showing that changes in gene expression resulting from protoplast isolation is essentially a wound response. Nevertheless, the induction of the two sets of genes differs in some respects: the PR mRNA level is identical in 1 mm leaf strips and in protoplasts, suggesting that the inducer is more diffusible. PR mRNAs accumulate simply after water infiltration in large explants even in unwounded leaf (Godiard *et al.*, 1990). In addition, the highest level of PR mRNAs is obtained by maintaining leaf strips in water or protoplasts in KCl+$MgSO_4$, while the culture medium partly inhibits this gene induction. This suggests that the mediators implicated in the induction of PR protein synthesis and in the re-entry into proliferation are different. These differences in the induction of different sets of genes are probably related to the various physiological responses that plants present after wounding, and which probably limit the consequences of this injury. The best analyzed reaction is the systemic induction of proteinase inhibitors (Pena-Cortes *et al.*, 1988; Ryan, 1987) and of other genes (Hildmann *et al.*, 1992), which makes the plant inedible for insects. It has been shown that oligosaccharides released by wounding have the potential to induce the systemic response although they do not migrate into the plant (Ryan and An, 1988). They induce a second messenger, a small protein named systemin (Pearce *et al.*, 1991; Ryan, 1992). In addition to systemic defense, plants present local defense reactions which limit infection of wounded tissue including accumulation of PR proteins and necrosis. Apart from these defense reactions, mechanisms able to repair damage (induction of cell growth and division) are activated. Signals which induce local responses have not yet been characterized, because the analysis of the wounded tissue is limited by the small number of reacting cells. Our study suggests that most genes activated during protoplast isolation are wound-induced, making these cells an attractive material for the analysis of the local wound reaction.

Experimental procedures

Plant materials

Nicotiana tabacum cell suspensions were derived from pith by Jouanneau *et al.* (Jouanneau and Tandeau de Marsac, 1973). They were subcultured every week by 10 × dilution. Three-day-old cultures of suspension cells, in the growth phase, contain dividing cells (mitotic index approximately 5%) while 11-day-old cultures, in stationary phase, consist mostly of non-dividing cells (mitotic index < 0.01%). Tobacco plants were grown in a greenhouse under controlled conditions. Mature tissues were collected from 3-month-old plants: fully expanded leaves, roots, stem and cortex stele. Young tissues, developing leaves and roots, were

collected from 2-week-old plants. Young flower buds (1 cm long) were also collected. Seeds in formation were taken from green fruits. Dry seeds were placed on nutritional medium and germinating seeds were collected 5 days later.

N. tabacum *cell suspension cDNA library*

Total RNA was extracted from frozen 7-day-old *N. tabacum* suspension cells as previously described (Grosset *et al.*, 1990b). Poly(A)$^+$ RNA was purified by chromatography on an oligo(dT) cellulose column. Double-stranded cDNAs were prepared using an Amersham cDNA Synthesis System Plus kit, and were ligated into the vector pTZ18R. *Escherichia coli* DH5-α cells were transformed with the library by the method of Hanahan (Hanahan, 1983).

Differential screening

The pTZ18R-cDNA library was plated at a low colony density and screened by differential hybridization. Duplicate Gene-Screen nylon-membrane (NEN) replicas were hybridized with ^{32}P-labeled single-stranded cDNAs synthesized from Poly(A)$^+$ RNA isolated either from cell suspensions in growth phase (3-day-old), stationary phase (11-day-old), or expanded leaves. Hybridizations were performed according to the instructions from the manufacturer. Selected cDNA clones isolated by this method were conserved in 50% glycerol.

Tobacco mesophyll protoplasts

Protoplasts were isolated from leaves of greenhouse tobacco plants (*N. tabacum* var. Maryland). Expanded leaves were sterilized with 15% sodium hypochloride for 20 min followed by 5 sec in 95% ethanol. They were washed three times for 10 min with sterile water and cut into thin strips of 1 mm width with a sterile razor blade. The strips were plasmolyzed for 10 min in 2.5% KCl, 1% $MgSO_4 \cdot 7H_2O$. This solution was replaced by another containing 11% mannitol, 0.4% glycine and 0.05% PATE (crude extract of Pectin Acid TransEliminase from *Bacillus polymyxa*) adjusted at pH 8.0. The leaf strips were infiltrated under vacuum but without shaking. After 90 min, the solution was replaced by 2.5% KCl, 1% $MgSO_4 \cdot 7H_2O$, 0.25% cellulase Onozuka10 (Yalkult Honsha Co.) and 0.02% cellulase Y.C. (Seichin Pharmaceutical Co.) adjusted at pH 5.7. Leaf strips were infiltrated every 15 min during 90 min. Resulting protoplasts were collected by slow centrifugations and washed three times in KCl–$MgSO_4 \cdot 7H_2O$ solution. Finally, they were resuspended in W0.6 culture medium (Meyer and Abel, 1975) at 5×10^5 protoplasts ml^{-1} and cultivated in Petri dishes at 25°C and in the dark for 14 h.

DNA probes

Poly(A)$^+$ RNA was purified from suspension cells in growth (3-day-old) and stationary (11-day-old) phases, and from mature leaves. The first strand of cDNA was synthesized by incorporation of ^{32}P dCTP (Shaw *et al.*, 1984). These probes were used for library screening and for the analysis of positive clones on Southern blot. Cloned cDNAs were obtained by digestion of the corresponding plasmid with the appropriate restriction enzymes and each cDNA insert was separated from the vector on an agarose gel. Recovery and elution of each insert were carried out using Schleicher and Schuell NA 45 paper according to the instructions of Maniatis (Maniatis *et al.*, 1990).

Three thioredoxin h probes were used. The first is a cDNA obtained during the screening described during this study; it contains a complete ORF encoding a protein that we have named thio h1. Using this clone we have isolated a genomic clone which encodes a second thioredoxin that we have named thio h2 (Brugidou *et al.*, 1993) which differs significantly from the thio h1cDNA. A 240 bp fragment corresponding to the 3′ noncoding region of the thio h1cDNA was recovered and used as a specific probe of the thioredoxin h1 gene. The specific nucleotide probe of thioredoxin h2 was prepared by recovering a 468 bp fragment in the noncoding region of the corresponding gene. The *Arabidopsis* histone H4 clone (A748) was given by Dr Claude Gigot (Chaboute *et al.*, 1987). It contains a 196 bp fragment in the H4 coding region, which was excised and labeled by the multiprime technique.

All these nucleotide inserts were labeled by the multiprime technique (Amersham Kit and procedures). A wheat rDNA fragment containing the 25S RNA coding region (Gerlach and Bedbrook, 1979) was used as a control probe for the quantity of rRNA and was labeled by nick-translation technique (Amersham Kit).

Northern blot analysis

Total RNAs were extracted from different tissues by previously described methods (Grosset *et al.*, 1990b). Different quantities of total RNA depending on the materials (2 μg for protoplasts and 10 μg for suspension cells and tissues) were denatured and fractionated on denaturing gels as described by Meinkoth and Wahl (1984). RNAs were transferred on to Gene-Screen membranes and prehybridized for 2 h at 42°C with the following solution: 50% deionized formamide, 2× Denhardt's, 5× SSC, 1% SDS, 100 μg ml^{-1} tRNA yeast, 100 μg ml^{-1} polyU. The membranes were hybridized in the same solution, successively with each denatured probe at 42°C for 20 h. The membranes were washed twice with 2× SSC at room temperature for 10 min, and twice at 70°C with 2× SSC, 1% SDS for 20 min. A stringent wash was made at 70°C with 0.5× SSC, 1% SDS during 10 min. Hybridization to the heterologous *Arabidopsis* H4 histone probe was made at 37°C and without the stringent wash.

Nucleotide sequences

The cDNAs were cloned in pTZ18R by *Bam*HI linker ligation at the 3′ extremity and a polyC ligation at the 5′ extremity. Nucleotide sequences of the 3′ region were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with double-stranded DNA templates using the Amersham Multiwell system as indicated by the manufacturer. In contrast, the Klenow-polymerase could not copy the polyC strand of the 5′ extremity and the Sanger method could not be applied for the sequencing of these fragments. They are sequenced by the Maxam and Gilbert technique (Maxam and Gilbert, 1980) according to the protocol of D. Delcasso-Tremousaygue (Delcasso-Tremousaygue *et al.*, 1988).

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References

Adams, M.D., Kelley, J.M., Gocayne, J.D. *et al.* (1991) Complementary DNA sequencing: Expressed sequence tags and human genome project. *Science*, **252**, 1651–1656.

Bergsma, D.J., Eder, C., Gross, M. *et al.* (1991) The cyclophilin multigene family of peptidyl-prolyl isomerases. Characterization of three separate human isoforms. *J. Biol. Chem.* **266**, 23 204–23 214.

Betersmann, A., Vogt, K. and Follmann, H. (1981) Multiple thioredoxin from non-green plant cells. In *Thioredoxin: Structure and Functions* (Gadal, P., ed.). CNRS, pp. 30–39.

Bhat, G.B., Iwase, K., Hummel, B.C. and Walfish, P.G. (1989) Kinetic characteristics of a thioredoxin-activated rat hepatic and renal low-Km iodothyronine 5′ deiodinase. *Biochem. J.* **258**, 785–792.

Bignon, C., Roux-Dosseto, M., Zeigler, M.A., Wicha, M.S. and Martin, P.M. (1992) cDNA cloning and genomic analysis of a new multigene family sharing common phylogenic and expression profiles with the lamin receptor gene. *Biochem. Biophys. Res Commun.* **184**, 1165–1172.

Brugidou, C., Marty, I., Chartier, Y. and Meyer, Y. (1993) The *Nicotiana tabacum* genome encodes two cytoplasmic thioredoxin genes which are differently expressed. *Mol. Gen. Genet.* **238**, 285–290.

Chaboute, M.E., Chaubet, N., Phillips, G. and Gigot, G. (1987) Genomic organization and nucleotides sequences of two histones H3 and two histones H4 genes of *Arabidopsis thaliana*. *Plant Mol. Biol.* **8**, 179–191.

Cooke, R. and Meyer, Y. (1981) Hormonal control of tobacco protoplast nucleic acid metabolism during *in vitro* culture. *Planta*, **152**, 1–7.

Criqui, M.C. (1992) *Evènements moléculaires précoces dans les protoplastes issus de cellules de mésophylle de* Nicotiana sylvestris. Thesis. Strasbourg: University Louis Pasteur.

Criqui, M.C., Jamet, E., Parmentier, J., Marbach, J., Dur, A. and Fleck, J. (1992) Isolation and characterization of a plant cDNA showing homologies to animal glutathione peroxidases. *Plant Mol. Biol.* **18**, 623–627.

Criqui, M.C., Plesse, B., Durr, A., Marbach, J., Parmentier, Y. and Fleck J. (1993) Characterization of genes precociously, strongly and transiently expressed in protoplasts of *Nicotiana sylvestris*.

Decottignies, P., Schmitter, J.M., Jacquot, J.P., Dutka, S., Picaud, A. and Gadal, P. (1990) Purification, characterization, and complete amino acid sequence of a thioredoxin from a green alga, *Chlamydomonas reinhardtii*. *Arch. Biochem. Biophys.* **280**, 112–121.

Decottignies, P., Schmitter, J.M., Dutka, S., Jacquot, J.P. and Miginiac-Maslow, M. (1991) Characterization and primary structure of a second thioredoxin from the green alga, *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* **198**, 505–512.

Delcasso-Tremousaygue, D., Grellet, F., Panabières, F., Ananiev, E.D. and Delseny, M. (1988) Structural and transcriptional characterization of the external spacer of a ribosomal RNA nuclear gene from a higher plant. *Eur. J. Biochem.* **172**, 767–776.

Dowdy, S.F., Lai, K.M., Weissman, B.E., Matsui, Y., Hogan, B.L. and Stanbridge, E.S. (1991) The isolation and characterization of a novel cDNA demonstrating an altered mRNA level in nontumorigenic Wilms' microcell hybrid cells. *Nucl. Acids Res.* **19**, 5763–5769.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. and Schmid, F.X. (1989) Cyclophilin and peptidyl-propyl *cis–trans* isomerase are probably identical proteins. *Nature*, **337**, 476–478.

Flamigni, F., Marmiroli, S., Caldarera, C.M. and Guarnieri, C. (1989) Involvement of thiol transferase- and thioredoxin-dependent system in the protection of "essential" thiol groups of ornithine decarboxylase. *Biochem. J.* **259**, 111–115.

Fleck, J., Durr, A., Lett, M.C. and Hirth, L. (1979) Changes in protein synthesis during the initial stage of life of tobacco protoplasts. *Planta*, **145**, 279–285.

Florencio, F.J., Yee B.C., Johnson, T.C. and Buchanan, B.B. (1988) An NADP/thioredoxin system in leaves: purification and characterization of NADP-thioredoxin reductase and thioredoxin h from spinach. *Arch. Biochem. Biophys.* **266**, 496–507.

Gasser, C., Gunning, D.A., Budelier, K.A. and Brown, M. (1990) Structure and expression of cytosolic cyclophilin/peptidyl-propyl *cis–trans* isomerase of higher plants and production of active tomato cyclophilin in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **87**, 9519–9523.

Genschik, P., Parmentier, Y., Criqui, M.C. and Fleck, J. (1990) Sequence of a ubiquitin carboxyl extension protein of *Nicotiana tabacum*. *Nucl. Acids Res.* **18**, 4007–4007.

Gerlach, W.L. and Bedbrook, J.R. (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucl. Acids Res.* **7**, 1869–1885.

Godiard, I., Ragueh, F., Froissard, D., Leguay, J.J., Grosset, J., Chartier, Y., Meyer, T. and Marco, Y. (1990) Analysis of the synthesis of several Pathogenesis-Related proteins in tobacco leaves infiltrated with water and with compatible and incompatible isolates of *Pseudomonas solanacearum*. *Mol. Mol. Plant–Microbe Interact.* **3**, 207–213.

Grippo, J.F., Holmgren, A. and Pratt, W.B. (1985) Proof that the endogenous, heat stable glucorticoid receptor activating factor is thioredoxin. *J. Biol. Chem.* **260**, 93–97.

Grosset, J., Meyer, Y., Chartier, Y., Kaufmann, S., Legrand, M. and Fritig, B. (1990a) Tobacco mesophyll protoplasts synthesize 1,3-b-glucanase, chitinases and 'osmotins' during *in vitro* culture. *Plant Physiol.* **92**, 520–527.

Grosset, J., Marty, I., Chartier, Y. and Meyer, Y. (1990b) mRNAs newly synthesized by mesophyll protoplasts are wound-inducible. *Plant Mol. Biol.* **15**, 485–496.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580.

Hildemann, Th., Ebneth, M., Pena-Cortes, H., Sanchez-Serrano, J.J., Willmitzer, L. and Prat, S. (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. *Plant Cell*, **4**, 1157–1170.

Homlgren, A. (1985) Thioredoxin. *Ann. Rev. Biochem.* **54**, 237–271.

Jamet, E., Durr, A., Parmentier, Y., Criqui, M.C. and Fleck, J. (1990) Is ubiquitin involved in the dedifferentiation of higher plant cells. *Cell Diff. Devel.* **29**, 37–46.

Jenkins, M.K., Schwartz, R.H. and Pardoll, D.M. (1988) Effect of cyclosporin on T cell development and clonal deletion. *Science*, **241**, 1655–1658.

Johnson, T.C., Cao, R.Q., Kung, J.E. and Buchanan, B.B. (1987a) Thioredoxin and NADP-thioredoxin reductase from cultured carrot cells. *Planta*, **171**, 321–331.

Johnson, T.C., Wada, K., Buchanan, B.B. and Holmgren, A. (1987b) Reduction of purothionin by the wheat seed thioredoxin system. *Plant Physiol.* **85**, 446–451.

Jouanneau, J.P. and Tandeau de Marsac, N. (1973) Stepwise effects of cytokinin activity and DNA synthesis upon mitotic cycle events in partially synchronized tobacco cell. *Exp. Cell Res.* **77**, 167–174.

Kamo, M., Tsugita, A., Wiessner, C., Wedel, N., Bartling, D., Herrmann, R.G., Aguilar, F., Gardet-Salvi, L. and Schürmann, P. (1989) Primary structure of spinach chloroplast thioredoxin F: protein sequencing and analysis of complete cDNA clones for spinach chloroplast. *Eur. J. Biochem.* **154**, 197–203.

Kobrehel, K., Yee, B.C. and Buchanan, B.B. (1991) Role of the NADP thioredoxin system in the reduction of alpha amylase and trypsine inhibitor proteins. *J. Biol. Chem.* **266**, 6135–6140.

Lebrun, M. and Freyssinet, G. (1991) Nucleotide sequence and characterization of a maize cytoplasmic ribosomal protein S11 cDNA. *Plant Mol. Biol.* **17**, 265–268.

Liboz, T. (1990) *Structure et expression des gènes codant pour le facteur d'élongation de la traduction EF-1 alpha chez* Arabidopsis thaliana. Thesis. University of Toulouse.

Liboz, T., Bardet, C., Le Van Thai, A., Axelos, M. and Lescure, B. (1989) The four members of the gene family encoding the *Arabidopsis thaliana* translation elongation factor EF-1α are actively transcribed. *Plant Mol. Biol.* **14**, 107–110.

Maeda, K., Tsugita, A., Dalzoppo, D., Vilbois, F. and Schürmann, P. (1986) Further characterization and amino acid sequence of m-type thioredoxins from spinach chloroplasts. *Eur. J. Biochem.* **154**, 197–203.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1990) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor University Press.

Marcus, F., Chamberlain, S.H., Chu, C., Masiarz, F.R., Shin, S., Yee, B.C. and Buchanan, B.B. (1991) Plant thioredoxin-H – an animal-like thioredoxin occurring in multiple cell compartments. *Arch. Biochem. Biophys.* **287**, 195–198.

Marivet, J., Frendo, P. and Burkard, G. (1992) Effects of abiotic stresses on cyclophilin gene expression in maize and bean and sequence analysis of bean cyclophilin cDNA. *Plant Sci.* **84**, 171–178.

Marty, I. and Meyer, Y. (1991) Nucleotide sequence of a cDNA encoding a tobacco thioredoxin. *Plant Mol. Biol.* **17**, 143–147.

Marty, I. and Meyer, Y. (1992) cDNA nucleotide sequence and expression of tobacco cytoplasmic ribosomal protein L2 gene. *Nucl. Acids Res.* **20**, 1517–1522.

Maxam, A.M. and Gilbert, W. (1980) Sequencing with base-specific chemical cleavages. *Methods Enzymol.* **65**, 499–560.

Meinkoth, J. and Wahl, G. (1984) Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* **138**, 267–284.

Meyer, Y. and Abel, W.O. (1975) Budding and cleavage division of tobacco mesophyll protoplast in relation to pseudo-wall and wall formation. *Planta*, **125**, 253–262.

Meyer, Y. and Cooke, R. (1979) Time course of hormonal control of the first mitosis in tobacco mesophyll protoplasts cultivated *in vitro*. *Planta*. **147**, 181–185.

Müller, E.G.D. (1991) Thioredoxin deficiency in yeast prolongs S phase and shortens the G1 interval of the cell cycle. *J. Biol. Chem.* **266**, 9194–9202.

Müller, M., Viro, M., Balke, C. and Kloppstech, K. (1980) Polyadenylated mRNAs for the light-harvesting chlorophyll *a/b* protein. *Planta*, **148**, 444–447.

Nagata, T. and Takebe, I. (1971) Plating of isolated tobacco mesophyll protoplasts on agar medium. *Planta*, **99**, 12–20.

Pearce, G., Strydom, D., Johnson, C. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science*, **253**, 895–898.

Pena-Cortes, H., Sanchez-Serrano, J., Rocha Sosa, M. and Willmitzer, L. (1988) Systemic induction of proteinase-inhibitor II gene expression in potato plants by wounding. *Planta*, **174**, 84–89.

Pokalsky, A.R., Hiatt, W.R., Ridge, N., Rasmussen, R., Houk, C.M. and Shewmaker, C.K. (1989) Structure and expression of elongation factor-1a in tomato. *Nucl. Acids Res.* **17**, 4661–4673.

Pouteau, S., Huttner, E., Grandbastien, M.A. and Caboche, M. (1992) Specific expression of the tobacco Tnt1 retrotransposon in protoplasts. *EMBO J.* **10**, 1911–1918.

Reichard, P. (1988) Interactions between deoxyribonucleotide and DNA synthesis. *Ann. Rev. Biochem.* **57**, 349–374.

Rivera-Madrid, R., Marinho, P., Chartier, Y. and Meyer, Y. (1993) Nucleotide sequence of an *Arabidopsis thaliana* cDNA clone encoding a homologue to a suppressor of Wilm's Tumor. *Plant Physiol.* **102**, 329–330.

Ryan, C.A. (1987) Oligosaccharide signalling in plants. *Ann. Rev. Cell. Biol.* **3**, 295–317.

Ryan, C.A. (1992) The search for the proteinase inhibitor-inducing factor, PIIF. *Plant Mol. Biol.* **19**, 123–133.

Ryan, C.A. and An, G. (1988) Molecular biology of wound-inducible proteinase inhibitors in plants. *Plant Cell Env.* **11**, 345–349.

Sanger, F., Nicklen, S. and Couson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Schreiber, S. (1992) Immunophilin-sensitive protein phosphatase action in cell signaling pathway. *Cell*, **70**, 365–368.

Shaw, P.H., Carneiro, M. and Schibler, U. (1984) Rapid size determination of mRNAs complementary to cloned DNA sequences: plaque and colony hybrid selection of cDNA. *Gene*, **29**, 77–85.

Shewmaker, C.K., Ridge, N.P., Pokalsky, A.R., Rose, R.E. and Hiatt, W.R. (1990) Nucleotide sequence of an EF-1α genomic clone from tomato. *Nucl. Acids Res.* **18**, 4276.

Steel, L.F., Smyth, A. and Jacobson, A. (1987) Nucleotide sequence and characterization of the transcript of a *Dictyostelium* ribosomal protein gene. *Nucl. Acids Res.* **15**, 10 285–10 298.

Takahashi, Y. and Nagata, T. (1992) parB: an auxin-regulated gene encoding glutathione S-transferase. *Proc. Natl Acad. Sci. USA*, **89**, 56–59.

Takahashi, Y., Kuroda, H., Tanaka, T., Machida, Y., Takebe, I. and Nagata, T. (1989) Isolation of an auxin-regulated gene cDNA expressed during the transition from G0 to S phase in tobacco mesophyll protoplasts. *Proc. Natl Acad. Sci. USA*, **86**, 9279–9283.

Ursin, V.M., Irvine, J.M., Hiatt, W.R. and Shewmaker, C.K. (1991) Developmental analysis of elongation factor-1α expression in transgenic tobacco. *Plant Cell*, **3**, 583–591.

Van Langendonckt, A. and Vanden Driessche (1992) Isolation and characterization of different forms of thioredoxins from the green alga *Acetabularia mediterranea*: Identification of an NADP/thioredoxin system in the extrachloroplastic fraction. *Arch. Biochem. Biophys.* **292**, 156–164.

Vernet, T., Fleck, J., Durr, A., Fritsch, M., Pinck, M. and Hirth, L. (1982) Expression of the gene coding for the small subunit of ribulosebiphosphate carboxylase during differentiation of tobacco plant protoplasts. *Eur. J. Biochem.* **126**, 489–494.

Vijayasarathy, S., Ernest, I., Itzhaki, J., Sherman, D., Mowatt, M., Michels, P. and Clayton, C. (1990) The genes encoding fructose biphosphate aldolase in *Trypanosoma brucei* are interspersed with unrelated genes. *Nucl. Acids Res.* **18**, 2967–2975.

Vogt, K. and Follmann, H. (1986) Characterization of three different thioredoxins in wheat. *Biochim. Biophys. Acta*, **873**, 415–418.

Wedel, N., Clausmeyer, S., Herrmann, R.G., Gardet-Salvi, L. and Schürmann, P. (1992) Nucleotide sequence of cDNAs encoding the entire precursor polypeptide for thioredoxin m from spinach chloroplasts. *Plant Mol. Biol.* **18**, 527–533.

Wiegand, M., Ofenloch, H.B. and Eisele, K. (1989) Reactivation of the androgen receptor from murine preputial gland by thioredoxin or GSH. *J. Steroid Biochem.* **32**, 53–58.

Zelcer, A. and Galun, E. (1980) Culture of newly isolated tobacco protoplasts: Cell division and precursor incorporation following a transient exposure to coumarin. *Plant Sci. Lett.* **18**, 185–190.

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