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# Ubiquitin genes are differentially regulated in protoplast-derived cultures of *Nicotiana sylvestris* and in response to various stresses

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**Abstract**

Four ubiquitin mRNA size classes were found to be differentially regulated in mesophyll protoplast-derived cultures of *Nicotiana sylvestris*. Three mRNA families of 1.9, 1.6 and 1.35 kb were expressed as soon as protoplasts were isolated. The 1.9 and 1.6 kb size classes were transiently expressed during the first hours of culture, whereas the level of expression of the 1.35 kb size class was maintained as long as cells kept dividing. A 0.7 kb mRNA size class started to be expressed just before the first divisions were observed. cDNAs corresponding to each of these families were isolated from a 6-h-old protoplast cDNA library and characterized. The 1.9, 1.6 and 1.35 kb mRNAs thus encode 7- or more, 6- and 5-mers, respectively, of ubiquitin whereas the 0.7 kb mRNAs encode a monomer of ubiquitin fused to a carboxyl extension protein of 52 amino acids. The expression of ubiquitin genes was studied, using probes specific for each of these transcript families, during protoplast culture and, for comparison, after various stresses including heat shock, $HgCl_2$ treatment, a viral infection giving rise to a hypersensitive reaction, and an *Agrobacterium tumefaciens* infection which resulted in tumour formation. The 1.9 and 1.6 kb mRNA size classes were found to be stress-regulated, the 0.7 kb mRNA size class developmentally regulated and the 1.35 kb size class both stress- and developmentally regulated.

## *Introduction*

Protoplasts represent an attractive model to study the molecular mechanisms responsible for one of the most intriguing properties of plant cells: totipotency. In this regard, an easy-to-handle system is provided by protoplasts isolated from leaves of *Nicotiana sylvestris*. Indeed, once isolated and incubated in appropriate culture medium, these protoplasts are able to regenerate a cell wall and to re-enter the cell cycle within a few hours. Cells from just fully expanded leaves are in a quiescent

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers M74101 (*N. sylvestris* 6PU11), M74156 (*N. sylvestris* 6PU4) and M74100 (*N. sylvestris* 6PUCEP52-7).

stage (G0 phase) and well differentiated towards the photosynthetic function. On the contrary, protoplasts progressively become undifferentiated cells and at the same time enter G1 phase and divide. The transition between these two physiological stages requires a precise orchestration of numerous processes including switching off the leaf programme of gene expression and settling a new programme, while fighting against the multiple stresses of the isolation procedure [22, 23]. Indeed, we recently analysed a cDNA library from 6-h-old protoplasts and found that 80% of the clones were related to stress whereas part of the programme of gene expression was related to cell division [14].

We showed that several ubiquitin mRNAs were induced at the time protoplasts were isolated [33]. Ubiquitin might have several roles in protoplasts considering all the changes undergone by these cells within a short time. Ubiquitin is a 76 amino acid polypeptide highly conserved in all eukaryotes. It displays multiple functions in the cell (for review, see [40]): it is involved in selective degradation of proteins [12, 49]; it has a role in chromatin structure and possibly in gene expression since histones H2A and H2B can be ubiquitinated [15]; it is part of the stress response [20]; and it participates in ribosome biogenesis [21]. A few proteins have been shown to be degraded by the ubiquitin pathway, i.e. phytochrome [31, 53], cyclin [27], the MYCN oncoprotein [13], and possibly the yeast $\alpha 2$ repressor [30].

Our study is now devoted to the regulation of ubiquitin gene expression in protoplasts and protoplast-derived cultures as a system to study both the reaction of the cell to stress and the transition from G0 to G1 phase. Four ubiquitin mRNA size classes were found to be differentially regulated during protoplast culture. cDNAs for representatives of each size class were isolated and characterized. Specific probes were designed to study the regulation of the expression of ubiquitin genes not only in protoplast cultures, but also in response to various stresses, or pathogen infections. We show that the 1.9 and 1.6 kb ubiquitin mRNAs are stress regulated, the 0.7 kb mRNAs developmentally regulated, and the 1.35 kb mRNAS are stress and developmentally regulated. The expression of 1.35 kb mRNA encoding genes is shown in more details using specific probes.

## Materials and methods

### *Plant material*

Seeds of *Nicotiana sylvestris* and *Nicotiana tabacum* var. Xanthi nc were kindly donated by SEITA (Institut Expérimental du Tabac, Bergerac, France). Plants were grown for 50 days in a greenhouse in a 12 h light/12 h dark cycle at 18–25 °C.

*In vitro* plantlets were grown in a 12 h light/12 h dark cycle at 20–24 °C in a medium composed of macroelements and FeEDTA [42], microelements without $FeCl_3$ [29], sucrose (10 g/l) and agar (8 g/l). They were subcultured every 6 weeks and upper leaves were taken after 3–4 weeks for preparation of protoplasts.

Protoplasts were isolated from just fully expanded leaves of *in vitro* plantlets. Leaves were sliced into thin strips. The following steps of protoplast preparation were as previously described [33], and protoplasts were cultured according to Nagy and Maliga [44]. Short preparations including 4 h incubation in enzyme solution were performed. The viability of protoplasts was tested with 0.01% fluorescein diacetate [34], and the presence of cellulose was detected with 0.01% calcofluor [43].

Cell suspension cultures of *N. tabacum* var. Xanthi nc, initiated from friable calli were grown and subcultured every 3 weeks in a medium after Nagy and Maliga [44] except that 2,4-dichlorophenoxyacetic acid, 6-benzylaminopurine and xylose were omitted and 20 g/l sucrose were added instead of 127 g/l.

Stressed leaf strips were obtained by slicing leaves into 1–2 mm wide strips. These were immersed for 24 h in plasmolysis medium [33].

Two fully expanded leaves at the top of *N. sylvestris* plants were inoculated with G-TAMV (green tomato atypical mosaic virus) [56]

by rubbing the upper surface with an abrasive pad soaked in virus suspension at a dilution to produce 200 to 250 local lesions per leaf.

One just fully expanded leaf at the top of *N. sylvestris* plants was sprayed with a 0.1% mercuric chloride solution.

Stems of *N. sylvestris* plants were infected with the *Agrobacterium tumefaciens* strain B6S3 producing tumours [57].

*Nucleic acid extraction and analysis*

DNA and RNA extraction and analysis were performed as previously described [32, 33]. The amount of nucleic acids was determined by OD measurement and checked by UV illumination after electrophoresis and ethidium bromide staining. The single modification was the hybridization buffer for RNA gel blots: 5 × SSPE (20 × SSPE is 3.6 M NaCl, 0.2 M $NaH_2PO_4$, 0.02 M $Na_2$-EDTA pH 7.7) 50% formamide, 5 × Denhardt's solution [16] (100 × Denhardt's solution is 2% Ficoll, 2% PVP, 2% BSA), 1% SDS and 50 μg/ml denatured salmon sperm DNA.

*Probes*

The integrity as well as the amount of RNAs was systematically checked by using a potato 25S rRNA probe [38]. A cDNA encoding a tobacco basic chitinase was a gift from Dr Meins [54]. Clone pHUb14-38 contains a 680 bp *Xho* I/*Pst* I fragment of a human ubiquitin cDNA spanning the ubiquitin coding region [59]. A *Pst* I/*Eco* RI fragment of clone UbCEP52 contains the sequence for a *Chlamydomonas reinhardii* 52 amino acids CEP and its 3′-untranslated region [8]. Specific *N. sylvestris* ubiquitin probes were designed as described in Results.

The probes were labelled with [α-$^{32}$P]-dATP and [α-$^{32}$P]-dCTP (3000 Ci/mmol, Amersham) by random primer labelling [19]. The conditions were adjusted to obtain specific activities of about $10 \times 10^8$ dpm/μg DNA for DNA gel blot analysis, and $2.5 \times 10^8$ dpm/μg DNA for RNA gel blot analysis and screening of the cDNA library.

*Screening of a cDNA library and DNA sequencing*

A 6-h-old protoplast cDNA library was constructed in phage λgt10 [14]. It was screened with the human ubiquitin cDNA pHUb14-38 [59]. Hybridizations were performed overnight at 42 °C in 5 × SSPE, 50% formamide, 5 × Denhardt's solution, 1% SDS and 50 μg/ml denatured salmon sperm DNA. Filters were subsequently washed in 2 × SSC and 0.1% SDS for 30 min at 42 °C, and twice in 0.2 × SSC and 0.1% SDS for 30 min at 42 °C.

Isolated clones were amplified, inserts were purified and subcloned into plasmid vector Bluescript (Stratagene) according to standard experimental procedures [39, 51]. They were sequenced on both strands by the dideoxynucleotide chain termination method [52] using T7 DNA polymerase.

## Results

*Ubiquitin genes are differentially regulated during protoplast culture*

As a first step towards a detailed analysis of the regulation of ubiquitin gene expression, we followed their transcription in freshly isolated protoplasts after a 4 h enzymatic digestion (time 0 h), and in the derived culture after 1, 2 and 4 days. As controls, RNAs were extracted from the just fully expanded leaves from which protoplasts were isolated, from 7-day-old cell suspension cultures and from leaf strips immersed for 24 h in the protoplast isolation medium devoid of enzymes. A mRNA gel blot was performed and hybridized to the human ubiquitin cDNA pHUb14-38 carrying sequences of the coding region [59] (Fig. 1). All the observations reported below were confirmed after hybridization of the same blot to the specific probes described in the following section (not shown).

As reported earlier [33], three mRNA size classes of 1.9, 1.6 and 1.35 kb appeared as soon as protoplasts were isolated, while these mRNAs were detected at a much lower level in leaf strips

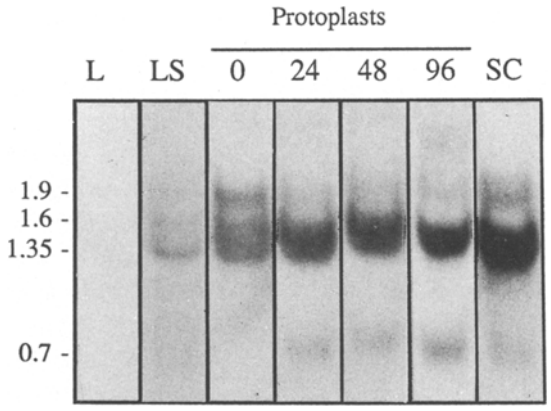


*Fig. 1.* Expression of ubiquitin genes in protoplasts and protoplast-derived cultures. Total RNAs were extracted from just-fully-expanded leaves (L), leaf strips immersed in protoplast isolation medium devoid of enzymes for 24 h (LS), protoplasts right after a 4 h isolation procedure (0), and after 24 h, 48 h and 96 h of culture, and a 7-day-old suspension culture (SC). 5 μg RNAs per sample were electrophoresed through an agarose-formaldehyde gel, transferred to a nylon membrane and probed with a labelled human ubiquitin cDNA (pHUb14-38). The sizes of the mRNAs are indicated in kb.

(LS). Low amounts of 1.35 kb mRNAs were detected in just-fully expanded leaves (L) after overexposure of the autoradiogram (not shown). The transcription of ubiquitin genes changed during protoplast culture: the 1.9 and 1.6 kb mRNAs rapidly decreased, the 1.9 kb mRNAs being maintained at a low level in cell suspension cultures. On the contrary, the 1.35 kb mRNAs were maintained at a high level, while the 0.7 kb mRNAs were detected after 1 day of culture and their amount kept increasing during the time culture was followed. The appearance of the 0.7 kb mRNAs preceded the observation of the first divisions: in this experiment, 15% of protoplasts were dividing after 3 days and 40% after 4 days.

*Three mRNA size classes encode polyubiquitins, the fourth one encodes a ubiquitin monomer fused to a carboxyl extension protein*

In order to characterize ubiquitin genes expressed in freshly-isolated protoplasts, a cDNA library from 6-h-old protoplasts [14] was screened. Two probes were used: the insert of the human cDNA pHUb14-38 [59], and the insert of a *Chlamydomonas reinhardii* cDNA carrying the sequence for a 52 amino acid carboxyl extension protein (CEP52) [8]. Positive clones were analysed by restriction mapping and quick sequencing of the ends. They were named 6P clones since they originated from the 6-h-old protoplast cDNA library.

Out of 1600 recombinant phages, 18 clones were selected with the human probe. They were sorted in three groups according to the number of encoded ubiquitin monomers: the longest cDNA of each of these groups is schematized in Fig. 2. Most clones were incomplete at their 5′ end probably because of inverted repeats in coding regions of ubiquitin mRNAs forming snap back loop structures which are self-primed during cDNA synthesis [2]. For these clones, the number of encoded ubiquitin monomers could be determined by comparing their length to the size of the corresponding mRNAs. 6PU1 encoded a 7- or 8-mer of ubiquitin, 6PU11 a 6-mer, 6PU18 and 6PU4 5-mers of ubiquitin. The number of ubiq-

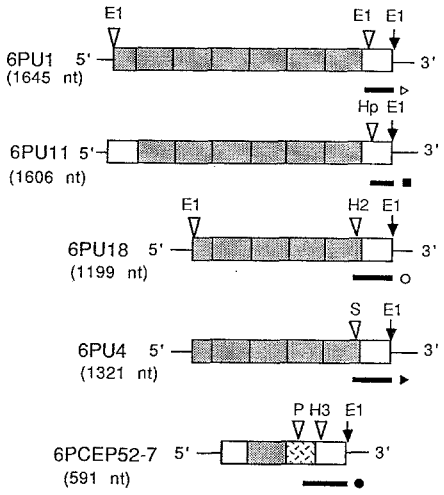


*Fig. 2.* Schematic representation of ubiquitin cDNAs isolated from a 6-h-old protoplast cDNA library. Shaded portions represent ubiquitin monomers, the speckled part a 52 amino acid carboxyl extension protein, and the white boxes the 5′- and 3′-untranslated regions. Horizontal bars indicated by symbols represent the fragments used as probes. Open arrows indicate restriction sites present in the cDNA sequences and black arrows the restriction sites present in the vectors used to prepare specific probes spanning 3′-untranslated regions. E1, *Eco* RI; H2, *Hind* II; Hp, *Hpa* I; P, *Pst* I; S, *Sal* I.

uitin monomers of the 6PU4 clone was confirmed by sequencing of the corresponding gene (P. Genschik *et al.*, unpublished results). On the other hand, the 6PU4 clone had a poly(A) tail of 48 nucleotides whereas the other clones were incomplete at their 3′ end.

Out of 50000 recombinant phages, 7 clones gave a positive signal with the CEP52 probe. All these clones had an identical sequence and encoded a ubiquitin monomer fused to a carboxyl extension protein of 52 amino acids schematized in Fig. 2 (6PCEP52-7). It should be noted that the CEP52 was already described as a constituent of the large ribosomal subunit in yeast [21].

The complete nucleotide sequences of cDNAs 6PU11, 6PU4 and 6PCEP52-7 are depicted in Figs. 3 and 4A. The repeats of 228 nucleotides encoding ubiquitin monomers were aligned below the first one taken as an arbitrary reference. Homologies were found to range between 80 to 90%. In all cases, these differences in nucleotide sequences corresponded to silent mutations.

The amino acid sequence of *N. sylvestris* ubiquitin is identical to that of higher-plant ubiquitins already described [4, 7, 11, 24]. It was previously noticed that higher-plant and yeast ubiquitins differ from the animal sequence at three positions (19, 24 and 28 for yeast or 57 for plants), and that two of the differences resulted in identical amino acids in yeast and plants [24]. All the *N. sylvestris* ubiquitin genes we have studied encode a unique non-ubiquitin residue at the 3′ end of the last ubiquitin repeat. It was a phenylalanine as reported for *Arabidopsis thaliana* [7] and for one sunflower ubiquitin gene [4]. Concerning the CEP52 amino acid sequence of the 6PCEP52-7 clone, it showed a homology of 94% to *A. thaliana* CEP52 [9], of 86% to *C. reinhardii* CEP52 [8] and of 78% to yeast CEP52 [47]. An alignment of amino acid sequences is given in Fig. 4B.

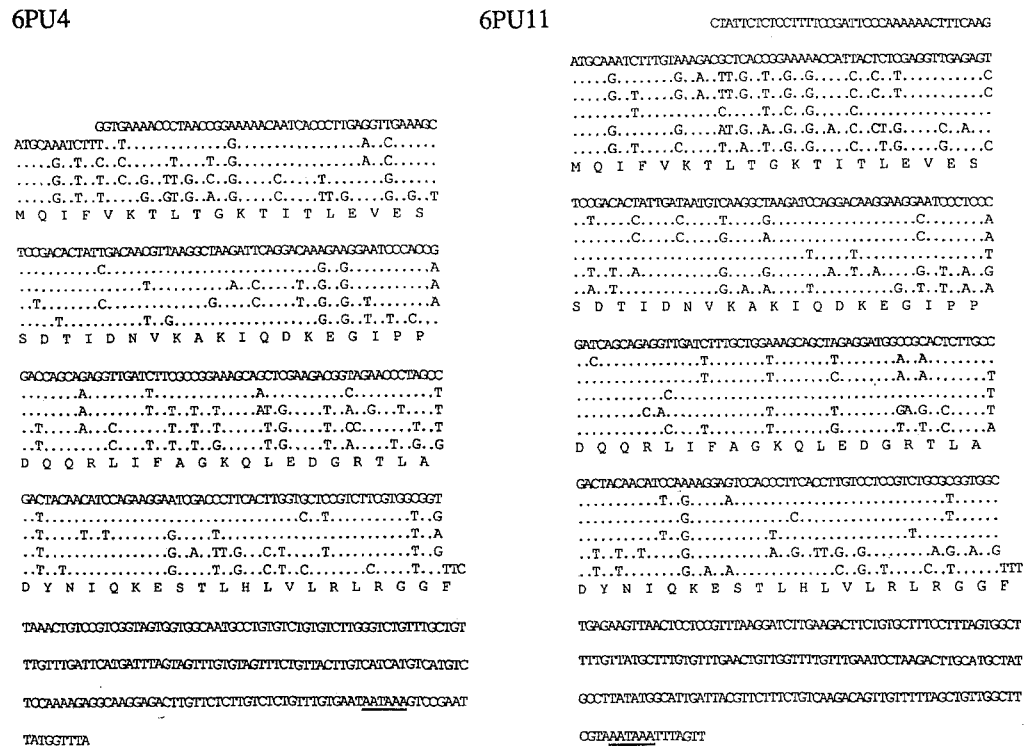


*Fig. 3.* Nucleotide and derived amino acid sequences of two polyubiquitin cDNAs (6PU4, 6PU11). Nucleotide sequences of the ubiquitin repeats are aligned. Identical nucleotides are indicated by dots. Amino acid sequences are given in the one-letter code below the last ubiquitin repeat. Putative polyadenylation signals are underlined.

(A)

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                                                                    AGAGAAGAGTGCGCCGCAAA
ATGCAGATCTTCGTAAAAACCCTAACCGGGAAGACAATCACGCTCGAGGTTGAATCGAGCGACACCATTGACAATGTCAAAGCTAAGATTCAAGACAAAGAAGGTATTCCACCG
M  Q  I  F  V  K  T  L  T  G  K  T  I  T  L  E  V  E  S  S  D  T  I  D  N  V  K  A  K  I  Q  D  K  E  G  I  P  P

GACCAGCAGCGGTTGATCTTTGCCGGAAAGCAGCTCGAAGATGGACGTACTCTTGCTGATTATAACATCCAGAAAGAGTCAACTTTGCATTTGGTTTTGAGGCTTCGTGGAGGG
D  Q  Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  A  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G

ATTATTGAGCCTTCTCTGATGGCTTTGGCGAGGAAGTACAACCAGGATAAGATGATTTGCCGCAAGTGCTATGCTCGCCTGCATCCTCGTGCTGTTAACTGCAGAAGAAAAAAA
I  I  E  P  S  L  M  A  L  A  R  K  Y  N  Q  D  K  M  I  C  R  K  C  Y  A  R  L  H  P  R  A  V  N  C  R  R  K  K

TGTGGGCACAGCAACCAGCTGAGGCCAAAGAAGAAGATCAAGTAGGCGTGATGTCTTTTCTAAGCTTAAATCAATTTTGTGTGTTGCAGCTATATTGCCAGTCCGTTGTTTTTA
C  G  H  S  N  Q  L  R  P  K  K  K  I  K  *

CAGTTTTCAGTCCTGCTTCAACTTGATGTCATGAATAGACAAGCATTTGTTTTGAACATCTAATTATTGGATAAGATATCTTTGTGCATTCAATATATGTCTGTTTCGATGGAAA
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(B)

| | |
|---|---|
| *Nicotiana sylvestris* | IIEPSLMALARKYNQDKMICRKCYARLHPRAVNCRRKKCGHSNQLRPKKKIK |
| *Arabidopsis thaliana* | .....M.....K..... |
| *Chlamydomonas* | .....Q.....E.....K...K.S...T.....L. |
| Yeast | .....K...S...C..SV.....P...T...KR....T.....L. |

*Fig. 4.* Nucleotide and derived amino acid sequence of 6PCEP52-7 (A) and amino acid sequence comparisons of CEP52 of plants and yeast (B). The four cysteine residues forming the zinc finger structure are indicated by arrowheads. The putative nuclear translocation signal is underlined. Dots indicate identical amino acids.

The cysteine residues forming a zinc finger motif essential for binding of nucleic acids [3] are conserved, as well as the putative nuclear localization signal [17].

The amino acid sequence of *N. sylvestris* 6PCEP52-7 was also different from that of a clone we isolated from a genomic library of *N. tabacum* [25]. The CEP portion of the *N. tabacum* encoded fusion protein is more likely to be a 52 amino acid CEP since the additional sequence of 60 nucleotides inserted between codons for amino acids 24 and 25 of classical CEP52 is probably an intron as consensus recognition sites border it. *A. thaliana* CEP52 also exhibits an intron, but located at a slightly different position [9]. On the other hand, this clone turned out to encode a ubiquitin monomer having changed amino acids as compared to the consensus plant ubiquitin sequence: it is thus probably a pseudogene.

An interesting feature of all the ubiquitin encoding cDNAs we isolated was that they all had different 3′-untranslated regions (not shown). This fact allowed us to design probes specific for each of them. They are schematized and indicated by recognition symbols in Fig. 2. These probes were employed for RNA gel blots where RNAs extracted from 0-h-old protoplasts (P0) and 7-day-old cell suspension cultures were analysed (SC) (Fig. 5). Each of them revealed a different class of ubiquitin mRNAs. 6PU1 corresponded to the 1.9 kb, 6PU11 to the 1.6 kb, 6PU4 and 6PU18 to the 1.35 kb and 6PCEP52-7 to the 0.7 kb mRNAs. It should be noted that the 1.6 kb mRNAs were not detected with the 6PU11 probe

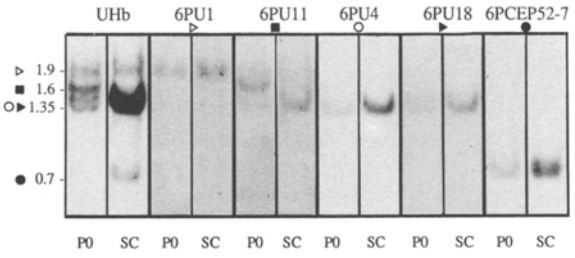


*Fig. 5.* Specific probes for each ubiquitin mRNA size class. Total RNAs were extracted from 6-h-old protoplasts (P0), and a 7 day-old cell suspension culture (SC). 5 $\mu$g per sample were electrophoresed in an agarose-formaldehyde gel and subsequently transferred to a nylon membrane. The same membrane was successively probed with the insert of the human pHUb14-38 (HUb) and with the 3′-untranslated regions of cDNAs 6PU1, 6PU11, 6PU4, 6PU18 and 6PCEP52-7 (for symbol explanation, see Fig. 2). Sizes of ubiquitin mRNAs are indicated in kb.

in 7-day-old cell suspension cultures. On the other hand, the autoradiogram showed a hybridization signal which actually corresponded to a hybridization to 1.35 kb mRNAs. An explanation would be that the same gene gives rise to two related mRNAs by differential splicing or that two highly related genes are differentially regulated since the two hybridization signals do not appear in the same conditions (compare P0 to SC). This collection of specific probes allowed us to better characterize the ubiquitin multigene family of *N. sylvestris* and to study the regulation of expression of some of its members.

### *Analysis of the ubiquitin multigene family of* Nicotiana sylvestris

Genomic DNA was isolated from *N. sylvestris* and restricted with *Eco* RI or *Hin*d III. There was one *Hin*d III site at the beginning of the 3′ untranslated region of cDNA 6PCEP52-7 (Fig. 2) and one *Eco* RI site at the very 5′ end of cDNAs 6PU1 and 6PU18 and in the 3′-untranslated region of cDNA 6PU1 (Fig. 2). DNA gel blots were performed and probed with the human cDNA probe and the specific *N. sylvestris* probes we designed and which are indicated by their specific recognition symbols from Fig. 2. Results are given in Fig. 6.

The human probe recognized 10 to 15 fragments depending on the restriction enzyme used. All the specific probes recognized at most two fragments.The same results were observed with *Eco* RV (data not shown). These results suggest that the corresponding genes are either unique per haploid genome, or present in multiple copies organized in clusters.

### *Polyubiquitin genes are regulated by stress*

We have previously shown that polyubiquitin genes were slightly activated in protoplasts due to the stress of the isolation procedure [33]. The question we then addressed was: how are ubiquitin genes activated in response to other stresses?

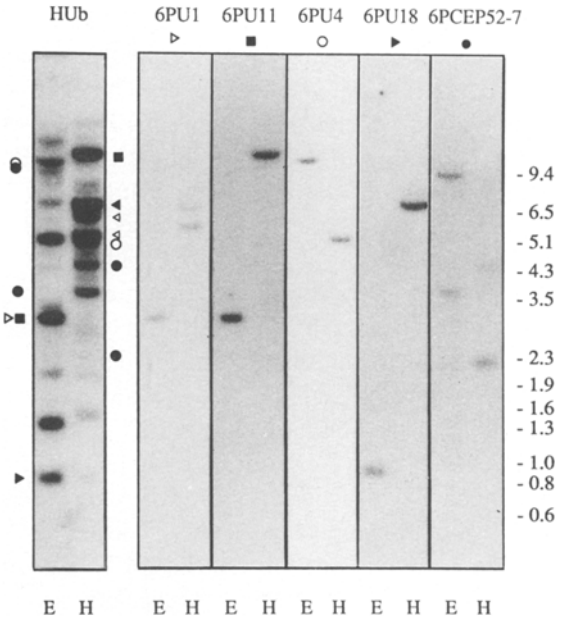


*Fig. 6.* Analysis of the ubiquitin multigene family of *N. sylvestris* using specific probes for each mRNA size class. Genomic DNA was restricted with *Eco* RI (E) or *Hin*d III (H) prior to electrophoresis in an agarose gel and subsequent transfer to a nylon membrane. The same membrane was successively probed with the human ubiquitin fragment (HUb) and with the 3′ untranslated regions of cDNAs 6PU1, 6PU11, 6PU4, 6PU18 and 6PCEP52-7 (for symbol explanation, see Fig. 2). Size markers are indicated in kb.

Having both the human probe and *N. sylvestris*-specific probes, we performed a detailed study of the regulation of polyubiquitin gene expression in response to heat shock, to mercuric chloride treatment and to viral infection giving rise to a hypersensitive reaction. The human probe spanning the ubiquitin coding region turned out to be a powerful tool since all the *N. sylvestris* ubiquitin coding regions we tested showed a strong preference for their own messenger RNA when taken as a probe for RNA gel blot analysis. On the contrary, the human probe revealed all *N. sylvestris* ubiquitin mRNAs in the same way.

Ubiquitin was already described as a heat shock protein in chicken [6], yeast [20] and maize [11]. To test the response of *N. sylvestris* to heat shock, plants were maintained for 20 min at 42 °C and total RNAs were extracted at various times after returning to 25 °C. Results from RNA

gel blot analyses are shown in Fig. 7. As previously described, control leaves mainly yielded 1.35 kb mRNAs at a basal level. The level of accumulation of polyubiquitin mRNAs was greatly enhanced after 20 min of heat shock. But already 30 min after transferring plants back to 25 °C, their abundance started to decrease and reached its basal level after about 180 min. Additional bands were revealed by the human ubiquitin probe. They might originate from other ubiquitin genes not yet characterized. The use of probes specific for each mRNA size class showed that only 1.9 kb (not shown) and 1.35 kb (Fig. 7) mRNAs were actively synthesized in response to heat shock. Hybridization of the RNA gel blot to the 6PU11 probe specific for the 1.6 kb mRNAs revealed no signal (not shown). The 6PU18 probe specific for 1.35 kb mRNAs revealed several bands (at least 3) ranging from 1.35 to 1.6 kb on that RNA gel blot. It may indicate that these mRNAs are differing in the length of their trailer region or of their poly(A) tail or that they could correspond to unspliced forms, as described for other genes [37]. On the contrary, the 6PU4 probe also specific for 1.35 kb mRNAs revealed a weak activation of the corresponding gene(s). Levels of 1.9 kb and 1.35 kb mRNAs accumulation were determined by densitometric analysis of the autoradiograms. They were found to be 8.5 and 3.5 fold higher respectively after 20 min of heat shock as compared to the control.

The second type of stress we tested was a chemical stress. Leaves were sprayed with a mercuric chloride solution and total RNAs were extracted at various times after the treatment. A weak but significant increase in the level of accumulation of 1.9 and 1.35 kb polyubiquitin transcripts was reproducibly observed (Fig. 8). The greatest accumulation was reached 6 h after the treatment. The effect of the treatment was monitored by the level of expression of the genes encoding a 3-hydroxy-3 methylglutaryl coenzyme A reductase [26].

Finally we looked at the accumulation of polyubiquitin mRNAs in response to a viral infection by G-TAMV (green tomato atypical mosaic virus) giving rise to a hypersensitive reaction (Fig. 9). Control plants were either non-treated (C) or mock-inoculated with sterile water (M). Test plants were inoculated with a suspension of G-

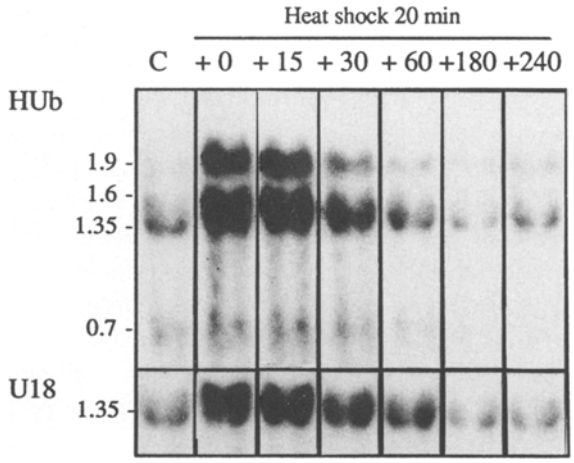


*Fig. 7.* Analysis of ubiquitin transcript levels in *N. sylvestris* plants heat shocked for 20 min at 42 °C. C stands for control leaves. Leaves from heat-shocked plants were harvested at various times after treatment from 0 min to 240 min. RNA gel blots were carried out and analysed as described in Materials and methods, with 5 μg of total RNAs loaded per lane. Hybridizations were successively performed on the same membrane with a human ubiquitin-coding region (HUb) and a probe specific for 6PU18 (U18). Sizes of ubiquitin mRNAs are indicated in kb.

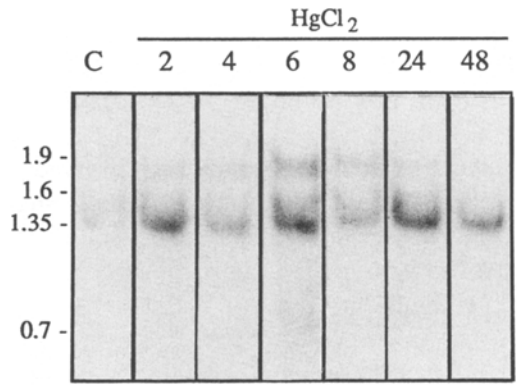


*Fig. 8.* Analysis of ubiquitin transcript levels in *N. sylvestris* leaves sprayed with a 0.1% mercuric chloride solution. C stands for control leaves. Treated leaves were harvested at various times after treatment from 2 h to 48 h. RNA gel blots were carried out and analysed as described in Materials and methods, with 5 μg of total RNAs loaded per lane. Hybridization was performed with a human ubiquitin-coding region (HUb). Sizes of ubiquitin mRNAs are indicated in kb.

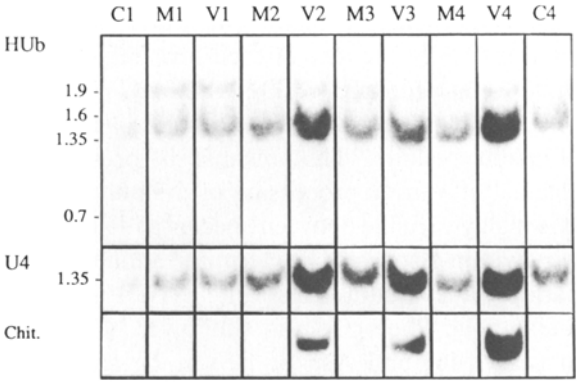


*Fig. 9.* Analysis of ubiquitin transcript levels in *N. sylvestris* leaves inoculated with G-TAMV and showing a hypersensitive reaction. C, M and V respectively stand for control leaves, leaves mock-inoculated with sterile water and virus-infected leaves. Samples were harvested 1 to 4 days after the beginning of the experiment. RNA gel blots were carried out and analysed as described in Materials and methods, with 5 $\mu$g of total RNAs loaded per lane. Hybridizations were successively performed on the same membrane with a human ubiquitin-coding region (HUb), a probe specific for 6PU4 (U4) and a cDNA coding for a basic chitinase (Chit). Sizes of ubiquitin mRNAs are indicated in kb.

TAMV adjusted to a concentration giving around 250 lesions per leaf. Again the accumulation of ubiquitin transcripts was not as strong as after heat shock and it was necessary to expose autoradiograms longer. The human probe already revealed a significant increase of the level of accumulation of ubiquitin 1.35 kb transcripts 2 days after inoculation which was also the time local lesions appeared. The amount of 1.35 kb mRNAs kept increasing during the time we followed the infection. This result was confirmed by using a probe specific for 1.35 kb 6PU4 mRNAs (Fig. 9). On the other hand, the response of the plant to this viral infection was monitored by examining the amount of transcripts of a basic chitinase, one of the pathogenesis-related (PR) proteins well known to be associated with plant defence reactions [10]. The level of accumulation of this mRNA was already very high 2 days after inoculation and kept increasing afterwards. It means that the reaction of the plants was very active.

*Expression of ubiquitin genes in dividing cells*

As mentioned above, due to the isolation procedure, protoplasts are highly stressed cells which are able to re-enter the cell cycle within a very short time. As shown in Fig. 1, the level of accumulation of ubiquitin transcripts varied during protoplast culture. The 1.35 kb polyubiquitin mRNAs were maintained at a high level in protoplast-derived cultures while the 0.7 kb mRNAs started to accumulate just before the first divisions occurred. We were interested in knowing how the corresponding genes were regulated in tissues where cells were actively dividing. We looked at the level of accumulation of ubiquitin transcripts in meristems and found a high level of 1.35 and 0.7 kb mRNAs as well as a significant amount of 1.6 kb mRNAs (data not shown). The second type of tissue we examined was stem tumours induced by *Agrobacterium tumefaciens* (Fig. 10). Control stems were inoculated with sterile water. Total RNAs were extracted at various times after inoculation and analysed. The response of the plant tissue to the inoculation was

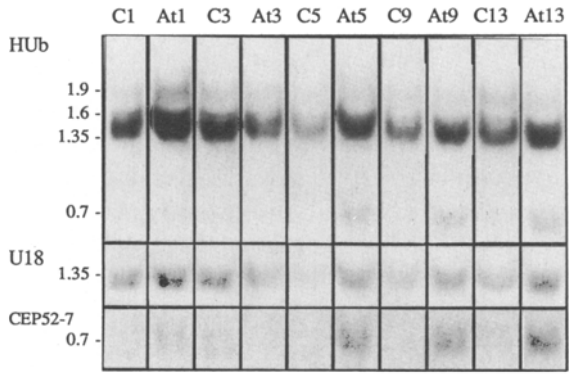


*Fig. 10.* Analysis of ubiquitin transcript levels in *N. sylvestris* stems infected by *Agrobacterium tumefaciens* during the tumorisation process. C and At respectively stand for control stems inoculated with sterile water and *A. tumefaciens*-infected stems. Samples were harvested 1 to 13 days after the beginning of the experiment. RNA gel blots were carried out and analysed as described in Materials and methods, with 5 $\mu$g of total RNAs loaded per lane. Hybridizations were successively performed on the same membrane with a human ubiquitin-coding region (HUb), a probe specific for 6PU18 (U18) and a probe specific for 6PCEP52-7 (CEP52-7). Sizes of ubiquitin mRNAs are indicated in kb.

a typical stress response after 1 day both in control and in *A. tumefaciens*-infected stems. The three classes of polyubiquitin mRNAs accumulated, the 1.35 kb mRNAs being predominant. After 5 days, polyubiquitin mRNA levels dropped back to the basal level in control stems. On the contrary, the accumulation of 1.35 kb mRNAs was maintained at a high level in infected stems and 0.7 kb mRNAs started to accumulate. This was also the time the tumor started to be visible. It should be noted that the amount of polyubiquitin mRNAs slowly increased with time in the control due to ageing of the tissues: 1.35 kb as well as 1.9 kb mRNAs were found at a relatively high level after 13 days (C13). This fact has also been observed in untreated senescent stems and leaves (not shown). But in all cases, the steady-state levels of 1.35 kb and 0.7 kb mRNAs were found to be higher in *A. tumefaciens* induced tumours than in control stems of the same age. These results were confirmed using probes specific for 6PU18 (1.35 kb mRNAs) and 6PCEP52-7 (0.7 kb mRNAs) (Fig. 10) and 6PU4 (1.35 kb mRNAs) (not shown).

## Discussion

Our results clearly show that several ubiquitin genes are differentially expressed in protoplasts and protoplast-derived cultures. Several cDNAs were isolated from a 6-h-old protoplast cDNA library and subsequently characterized. Together with yeast [47], *Dictyostelium discoideum* [45] and *A. thaliana* [7, 9], *N. sylvestris* is one of the organisms for which representatives of a number of ubiquitin mRNA size classes are now described.

Four size classes of ubiquitin transcripts are present in *N. sylvestris*: 1.9 kb, 1.6 kb, 1.35 kb and 0.7 kb. The 1.35 kb mRNAs are the most represented in protoplasts since they corresponded to half of the isolated cDNA clones. The 1.9 kb, 1.6 kb and 1.35 kb mRNAs encode 7-or more, 6- and 5-mers of ubiquitin, respectively, whereas 0.7 kb mRNAs encode a ubiquitin monomer fused to a 52 amino acid CEP. No cDNAs encoding the 80 amino acid CEP observed in other organisms including plants [40] have been isolated probably because of their low frequency in the 6-h-old protoplasts cDNA library. *N. sylvestris* polyubiquitins all end with a Phe as a non-ubiquitin residue. This residue is presumably cleaved off during processing of the polyprotein. It is highly variable between species and may even vary within a species. The terminal amino acid of barley polyubiquitin is also Phe [24], it is Glu in maize [11], Phe or Asn in sunflower [4, 5], Tyr in yeast [46] and Asn or Leu in *D. discoideum* [45]. The four RNA size classes we describe in *N. sylvestris* are also found in other plants like barley [24] and *A. thaliana* [7]. On the contrary, only three size classes are present in sunflower: 1.6 kb, 1.3 kb and 0.7 kb. The 1.6 kb transcripts are predominant and encode a 6-mer of ubiquitin [5]. Animal ubiquitin mRNAs can be longer, for example in *Drosophila* where a 4.4 kb transcript encodes a 18-mer [36] and in *Trypanosoma cruzi* where transcripts range in size from 0.65 kb to 12.5 kb, one of them encoding a 52-mer of ubiquitin [55]. Only one example of such unusually long ubiquitin mRNAs has been described for plants, this transcript appears after heat shock of maize seedlings and was interpreted as unspliced mRNAs [11].

Specific probes spanning 3′-untranslated regions of the cDNAs have been designed and used to study the genomic organization of ubiquitin genes. The ubiquitin multigene family probably comprises about twelve members in *N. sylvestris* which is comparable to what was described for barley [24], *A. thaliana* [7], flax [1] and sunflower [5]. The cDNAs we analysed are encoded by five different genes.

These specific probes were also used to study the regulation of the expression of several ubiquitin genes which could be related to stress and/or to development by examining their steady-state level of expression in various physiological conditions.

Due to the dual nature of protoplasts (stressed cells re-initiating their cell cycle) and to the diversity of ubiquitin functions in the cell (see Introduction), it was of importance to follow the expression of ubiquitin genes in protoplast-

derived cultures, in response to various stresses, and in actively dividing cells as a first approach towards the understanding of ubiquitin role(s) in protoplasts. This study allowed us to relate the expression of the 1.9 kb, 1.6 kb and 1.35 kb mRNA encoding genes to a stress state, whereas that of 1.35 kb and 0.7 kb mRNA encoding genes could be related to cell division. In all organisms studied so far, the expression of at least some polyubiquitin genes has been related to stress situations. It has especially been shown in the case of heat shock [6, 11, 20], starvation and presence of amino acid analogues [20], cold shock, presence of cadmium and inhibitors of protein synthesis [41] and wounding [33, 50]. In only one case, the steady-state level of a polyubiquitin gene was found to decrease after heat shock [7]. The activation of ubiquitin genes after mercuric chloride treatment has not been shown before, but it has probably to be linked to the effect of cadmium reported for *D. discoideum* as a general effect of heavy metals [41]. On the other hand, it is also the first time that the activation of ubiquitin genes after a plant viral infection is reported, although ubiquitinated structural proteins have been found in several plant virus particles [18, 28]. It was shown that ubiquitin gene expression is transcriptionally induced upon infection of hamster cells with herpes simplex virus [35]. Concerning the 0.7 kb ubiquitin fusion protein encoding mRNAs, they have been classically related to development since the extensions represent ribosomal proteins [21]. Our results on the expression of 0.7 kb mRNAs in protoplast-derived cultures, meristems and *A. tumefaciens*-induced tumours are in agreement with that finding. This has already been shown in the case of yeast [47], barley [24], *D. discoideum* [58] and *A. thaliana* [9]. In one case, *C. reinhardii*, the steady-state level of ubiquitin-CEP mRNAs was found to decrease after heat shock [48]. It was also shown to increase in potato tubers after wounding [50]. But this is not the case in *N. sylvestris*.

This is now the first time that the expression of some polyubiquitin genes, namely the 1.35 kb mRNA encoding genes, can be related to cell division. This is the case in protoplast-derived cultures, in meristems and in *A. tumefaciens*-induced tumours. Thus, the use of specific probes allows us to ensure that the same genes are indeed under a double control, stress and development.

It is interesting to note the differences in timing and relative levels of activation of polyubiquitin genes in response to the various stresses we applied to plants. The heat shock response occurs within the 20 min following the beginning of the treatment, is of very high intensity and transient as previously described [6, 11]. The reaction to mercuric chloride treatment occurs somewhat later and has its maximum around 6 h after treatment, whereas the response to wounding also occurs quite early but lasts for about 2 days [33, 50; this paper]. In both cases, the activation of ubiquitin genes is relatively weak. The activation of polyubiquitin genes in response to a viral infection becomes detectable only after 2 days, at which time local lesions appear. But it is probably a question of level of detection since only the cells around the points of virus entry participate in the defense reaction at the beginning of the infection process. However, there seem to be several levels of regulation of ubiquitin gene expression as exemplified by the difference between the effects of heat shock and mercuric chloride treatment. In both cases, all cells are participating in the defense reaction. Activation of ubiquitin genes by heat shock is then more likely to be a one-step mechanism since it occurs within such a short time, whereas it may require additional step(s) after a chemical treatment. On the other hand, 1.9 kb and 1.6 kb mRNAs are found in response to a chemical stress and to wounding. 1.6 kb mRNAs are not found after a heat shock, whereas 1.9 kb mRNAs accumulate at a very high level. On the contrary, the activation of 1.35 kb mRNA encoding genes seems to constitute a more general reaction of plants to any kind of aggression. However, it is interesting to note that the two sub-families we studied are not regulated in the same way. All these cases illustrate the complexity of the regulation of polyubiquitin gene expression.

Taken together, these results shed light on the

possible roles of ubiquitin in protoplasts. An essential function is probably exerted at the time protoplasts are isolated. As part of plant defense mechanisms, ubiquitin might be a key actor for protoplast recovery which seems to be a prerequisite for subsequent division [14]. But it should be noted that the expression of ubiquitin genes is not only related to the stress of the isolation procedure since it is only very weak after wounding [33]. The identification of target proteins at that stage will be an essential part of our future work to understand how far ubiquitin is involved in protein degradation during the process of dedifferentiation. Of course, another essential point is the regulation of the expression of ubiquitin genes in protoplasts. Polyubiquitin 1.35 kb mRNA-encoding genes could be essential in *N. sylvestris* since they are activated by all stress situations as well as during division. On the other hand, polyubiquitin 1.9 kb and 1.6 kb mRNA encoding genes are part of the immediate response of protoplast to the new environmental conditions: their expression is only transient. The availability of specific probes will allow us to isolate the corresponding genes. *Cis*- and *trans*-regulatory factors/elements active in protoplasts will be characterized. This should enable us to understand the complexity of the expression pattern of polyubiquitin genes in protoplasts especially since all of them encode the same polypeptide.

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