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Polyubiquitin gene expression and structural properties of the *ubi*4–2 gene in *Petroselinum crispum*

Petra Kawalleck, Imre E. Somssich, Michael Feldbrügge, Klaus Hahlbrock and Bernd Weisshaar*
Max-Planck-Institut für Züchtungsforschung, Abteilung Biochemie, Carl-von-Linne Weg 10, 5000 Köln 30, Germany (author for correspondence)*

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Abstract

Ubiquitin is an omnipresent protein found in all eukaryotes so far analysed. It is involved in several important processes, including protein turnover, chromosome structure and stress response. Parsley (*Petroselinum crispum*) contains at least two active polyubiquitin (*ubi*4) genes encoding hexameric precursor proteins. The deduced amino acid sequences of the ubiquitin monomers are identical to one another and to ubiquitin sequences from several other plant species. Analysis of the promoter region of one *ubi*4 gene revealed putative regulatory elements. In parsley plants, the *ubi*4 mRNAs were the predominant ubiquitin mRNAs and were present at comparable levels in all plant organs tested. In cultured parsley cells, high levels of ubiquitin gene expression remained unaffected by heat shock, elicitor or light treatment.

Introduction

Among all proteins known to date, ubiquitin is one of the most highly conserved throughout numerous eukaryotes investigated [46]. In all cases, the ubiquitin polypeptide consists of 76 amino acids, has a molecular mass of 8.5 kDa and an isoelectric point of 6.7. It is located intracellularly either free or linked to a variety of cytoplasmic, nuclear or integral membrane proteins. The best characterized role for ubiquitin is its covalent conjugation with target proteins as a recognition signal for protein turnover [28]. A complex sequence of enzymatic reactions attaches ubiquitin to other proteins, usually by covalent linkage to the ε-amino group of lysine residues at the target proteins. The resulting conjugate contains ubiquitin either as a monomer or as oligo ubiquitin chains which may form branched structures [27]. In addition, ubiquitination is involved in the heat-shock response and in the preservation of chromatin structure [23, 40]. There are various other, more or less firmly established, functions in several biological processes [19]. Examples involving ubiquitin from higher plants include the degradation of the P_{fr} form of phytochrome [26, 45] or effects of altered ubiquitin function on leaf morphology in tobacco [2].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X64344 (*ubi*4–1 cDNA) and X64345 (*ubi*4–2 gene)

The uibiquitin polypeptides are synthesised either with short, unrelated peptides of about 52 or 80 amino acids at the C-terminus (ubiquitin extension proteins; see e.g. [11]) or as polyproteins with varying numbers of ubiquitin monomers [43]. The ubiquitin extension proteins are involved in ribosome biogenesis [18], whereas the precursor polyproteins are split into the subunits to provide most of the ubiquitin found in eukaryotic cells. The last monomer of polyubiquitin peptides nearly always contains a C-terminal extension of one amino acid [40]. Maturation of the polyprotein involves removal of this tail, thus unmasking the C-terminal glycine-76 residue through which ubiquitin is joined to target proteins [19, 40].

Ubiquitin is encoded by multigene families, including genes coding for ubiquitin extension proteins and for ubiquitin polyproteins [40, 43]. The structure of a polyubiquitin gene was first described for the yeast *UBI4* locus [36, 37]. Polyubiquitin genes analysed so far comprise from 3 (*Phytophthora infestans* [38]) up to 52 (*Trypanosoma cruzi* [52]) direct repeats, each of which encodes one ubiquitin monomer. In plants, the number of repeats present in the polyubiquitin genes described to date varies from 5 in *Arabidopsis* (*Arabidopsis thaliana* [10]) to 6 in sunflower (*Helianthus annuus* [6]) and 7 in maize (*Zea mays* [13]).

In our attempts to functionally assign previously identified, elicitor-responsive as well as unresponsive genes [49], we found that a gene tentatively designated CON2 ('constitutively' expressed) encodes polyubiquitin. In this study, we analysed the expression of polyubiquitin mRNAs in parsley and describe the structure of one polyubiquitin gene in more detail.

Materials and methods

Cell cultures and plants

Parsley cell suspension cultures were propagated at 26 °C as described elsewhere [30]. Heat shock was applied continuously at 37 °C [55]. Parsley plants (cv. Hamburger Schnitt) were grown under greenhouse conditions for about one year. For RNA isolation, various organs from flowering plants were harvested, frozen in liquid nitrogen and stored until use at −80 °C.

cDNA and genomic libraries

The construction of the λgt11 cDNA [56] and the λEMBL-4 genomic [24] libraries have been described. Phage clones which were positive through at least three rounds of plaque hybridization were subjected to restriction mapping. cDNA inserts of λgt11 phages (λubi/1 to λubi/19) were subcloned via their engineered *Not* I sites into pSKII$^+$ (Stratagene) and the resulting plasmid clones were designated pubi/1 to pubi/19. The selected 2.8 kb *Eco* RI fragment from the genomic clone, gλubi-2, was subcloned into pKS$^-$.

Plasmids and hybridization probes

CON2b, CON2d, and CON2e are representative cDNAs for the 'constitutively' expressed parsley CON2 gene(s) [49]. The DNA for the ubi4 probe represents the entire *Eco* RI fragment of the CON2b plasmid (length as indicated in Fig. 1) and is identical to the probe designated con2, which was used in several earlier studies [32–34, 54, 56]. For screening of the λgt11 cDNA library, a fragment corresponding to the 3′ region of the cDNA was isolated from CON2b plasmid DNA. The probe 5′ubi4–2 was prepared from a 90 bp *Bgl* II fragment from the 5′ region of the 2.8 kb *Eco* RI fragment which had been subcloned into pSKII$^+$ (Stratagene). The gene-specific probes 3′ubi4–1 (180 bp in length) and 3′ubi4–2 (140 bp in length) were prepared from DNA fragments produced by PCR using the primers 4–1 dn, 4–2dn, and 4–2up as indicated in Figs. 1 and 5. For the 3′ubi4–1 probe, the second primer used was located in the vector close to the cloning site. Probes included for reference in the run-on assays were *prl*–1 cDNA [50, 51] and 18S rDNA [49].

Other materials

Oligonucleotides used for sequencing and PCR were synthesised on an Applied Biosystems DNA synthesizer and purified on Sephadex G-25 (Pharmacia) columns. Enzymes were purchased from Boehringer, Biolabs, BRL and Pharmacia, and radionucleotides were from Amersham-Buchler. DNA length standards were obtained from BRL.

Standard procedures

Conventional molecular biology techniques were carried out as described [41]. Hybridization experiments were conducted using Hybond N nylon membranes (Amersham-Buchler), and isolated DNA fragments were labelled by random priming [17] using premixed reagents (Boehringer). The filters were hybridized in 10% dextran sulphate, 1M NaCl, 50 mM Tris/HCl pH 7.5, 10 × Denhardt's solution and 1% SDS at 65 °C for 16 h, using 10^7 cpm of labelled probe and washed in 0.2 × SSC, 0.5% SDS at 60 °C (stringent conditions).

Nucleic acid isolation

RNA was isolated according to Weisshaar *et al.* [56] and genomic DNA from cultured parsley cells was prepared according to Schulze-Lefert *et al.* [44].

Sequence analysis

Sequencing was carried out using the dideoxy chain termination method [42], double-stranded plasmid DNA, and the T7 DNA sequencing kit (Pharmacia). Most of the sequencing was done by creating the appropriate subclones. Non-redundant parts of the 5′ sequences of *ubi*4–2, without useful restriction sites, were sequenced with the help of synthetic oligonucleotides. The sequencing reaction products were resolved on sequencing gels with the addition of 1 M sodium acetate to the bottom buffer (simplified salt gradient gels [47]). Except for the 5′ portion of the *ubi*4–1 cDNA sequence, which is unique to pubi/1, all sequences have been verified on two strands. Nucleotide and amino acid sequence analysis was performed on a VAX computer using the UWGCG program package [14].

Nuclear run-on transcription

The conditions for run-on transcription assays have been described [49]. In the experiments presented here, the amount of labelled RNA added was kept constant (10^7 cpm), because with nuclei from heat-shocked cells the amount of label yielded from an equal number of nuclei (corresponding to 200 μg DNA) was consistently about 3-fold lower than with nuclei from control cells. The amount of linearized recombinant plasmid DNA loaded onto the nitrocellulose filters was 1 μg per sample.

Rapid amplification of cDNA ends (RACE)

To map the 5′ end of the *ubi*4–2 RNA, a modification of the RACE protocol [20] was used. After a 'primer extension' reaction with 10 μg total RNA from untreated cultured cells using primer 1 (5′-CAAAGATCTGCATACCACCAC-3′) and subsequent A-tailing with terminal transferase, a PCR was performed using primer 2 (5′-GCGGATCCGACGGAGCACCAGATGG-3′) and a modified SK primer containing a *Hin*d III recognition sequence as well as 17 T residues at its 3′ end (5′-TCTAGAACTAGTGGATCAAGCTTTTTTTTTTTTTTT-3′). The products were electrophoretically size-separated, and DNA fragments of the expected size range (i.e. the part of the first ubiquitin repeat plus the 5′ untranslated region) were isolated. After a second PCR using the isolated fragments as template as well as primer 3 (5′-GCGGATCCATGTTGTAGTCAGCTAGG-3′) and the SK primer (without the T residues), the resulting products were gel-purified and cloned via the re-

striction sites introduced by the primers (*Spe* I and *Bam* HI). The anticipated annealing positions of the primers 1, 2 and 3 are indicated in Fig. 5. Obviously, because of the redundancy of the ubiquitin sequence, the primers can also anneal at different positions. To reduce this misannealing the primers were placed at relatively sequence-variable positions. Also, the size separation and the repeated use of different primers increased the probability for obtaining a specific product.

Results

Identification of cDNAs encoding ubiquitin

The longest representative of the previously isolated CON2 cDNAs (CON2b) [49] was sequenced. Comparison of the deduced amino acid sequence with GenBank data revealed that CON2b encodes 3 complete and one partial ubiquitin repeat units. In addition, it contains DNA complementary to the 3′-untranslated region as

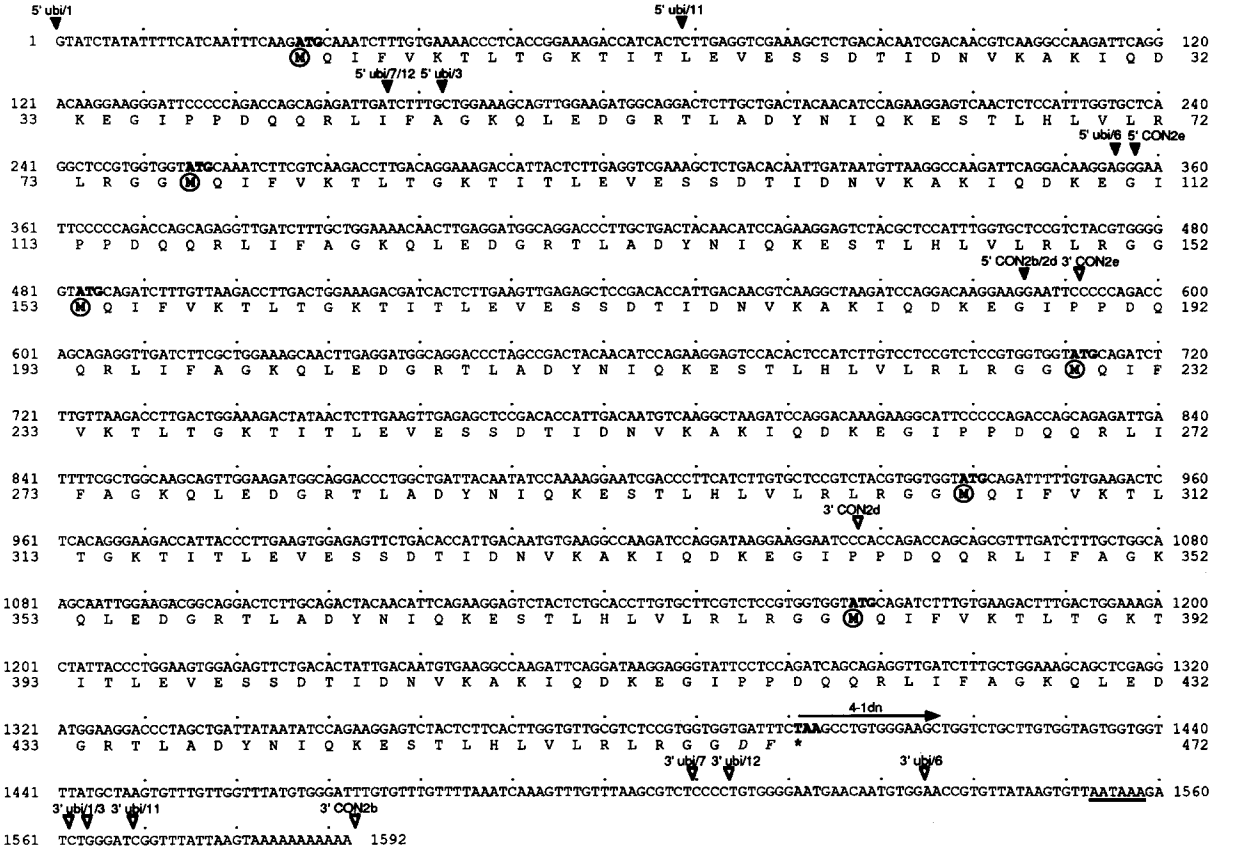

Fig. 1. Nucleotide and deduced amino acid sequence of the parsley *ubi*4–1 polyubiquitin cDNA. The 5′ ends (closed triangles) and 3′ ends (open triangles) of several cDNAs of identical sequence are indicated. ATG codons highlighted in bold correspond to the 5′ ends of the 6 repeat units, each of which encodes one ubiquitin monomer. The corresponding methionine residues are encircled. Amino acids extending from the last monomer are marked in italic style and the stop codon is marked by bold style. The position of the potential polyadenylation signal [29] is underlined and the arrow marks the annealing position of the oligonucleotide used to prepare the 3′ubi4–1 probe. Linker sequences added to the cDNA during cloning, such as the *Eco* RI site at the 3′ terminus of the CON2b cDNA, are not included.

well as a poly(A) tail. The parsley gene represented by this cDNA was designated *ubi*4–1.

Two other cDNAs, CON2d and CON2e, also encode ubiquitin and seem to correspond to internal *Eco* RI fragments of a polyubiquitin cDNA. These *Eco* RI ends, as well as the *Eco* RI site at the 5′ end of CON2b, are most likely due to incomplete methylation of *Eco* RI restriction sites during construction of the original cDNA library [15]. To obtain the missing 5′ end, new clones were isolated from a different λgt11 cDNA library [56]. The chance of detecting related cDNA clones was increased by using a fragment from the 3′part of the CON2b cDNA as a probe. Nine independent clones were plaque-purified and sequenced from both ends. Six of them (pubi/1, pubi/3, pubi/5, pubi/6, pubi/7 and pubi/11) contained sequences identical to CON2b (*ubi*4–1). The combined nucleotide sequences of the initial CON2 cDNAs and the 6 new *ubi*4–1 cDNAs, including part of the 5′-untranslated region, are shown in Fig. 1. Three related but non-identical cDNAs, lacking internal *Eco* RI sites (pubi/14, pubi/15, pubi/19), were derived from a very similar type of polyubiquitin mRNA representing a closely related gene that was designated *ubi*4–2.

CON2 was initially identified as a gene whose expression was not significantly affected by light or elicitor treatment of cultured parsley cells and hence has been used under these conditions as an internal control probe in RNA blot and nuclear run-on experiments [32–34, 54, 56]. We note that RNA blots always yielded only one signal in the 1.8 kb range, which we now interpret as the sum of several closely related mRNAs transcribed from different parsley *ubi*4 genes (see below).

Total ubiquitin gene expression is not affected by heat shock in parsley

To test whether heat shock affects the overall *ubi* gene expression in cultured parsley cells, nuclear run-on assays using nuclei from heat-treated and untreated cells were performed. As a control for efficient induction of the heat-shock response, ^{35}S-labelled proteins from a portion of the heat-treated cells were isolated and analysed for the presence of heat-shock proteins. The observed pattern of labelled proteins was similar to that described earlier for heat-shocked parsley cells [55]. Besides the *ubi*4–1 cDNA, *pr*1 and 18S rDNA probes were used as controls. The data presented in Fig. 2 demonstrate that the total expression of *ubi* genes in parsley cells was not affected by heat shock. The controls show that expression was slightly reduced for the 18S rRNA and drastically reduced for the *pr*1 genes. Essentially similar results were obtained at the mRNA level in RNA blot experiments (data not shown).

ubi*4 mRNAs are the most abundant ubiquitin mRNAs in parsley plants*

We also analysed *ubi* gene expression in parsley plants. In total RNA isolated from different plant organs, the 1.8 kb *ubi*4 mRNAs were the predominant type of ubiquitin-encoding mRNAs (Fig. 3). mRNAs differing considerably in size from 1.8 kb were not observed. Weak signals in the lower part of the autoradiogram, especially in RNA from flower stems, are due to background hybridization. The size of the *ubi*4 mRNA in whole plant tissue and cultured cells was identical. In all organs analysed, the polyubiquitin mRNAs were present in considerable amounts.

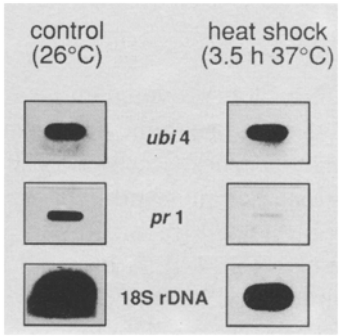

Fig. 2. Effect of heat shock on gene expression. Labelled run-on RNA was hybridized with filter-bound plasmid DNA detecting *ubi* and control (*pr*1 and 18S rRNA) transcripts. Autoradiographs of filters incubated with RNA from heat-shocked nuclei (right) and control nuclei (left) indicate the respective gene transcription rates.

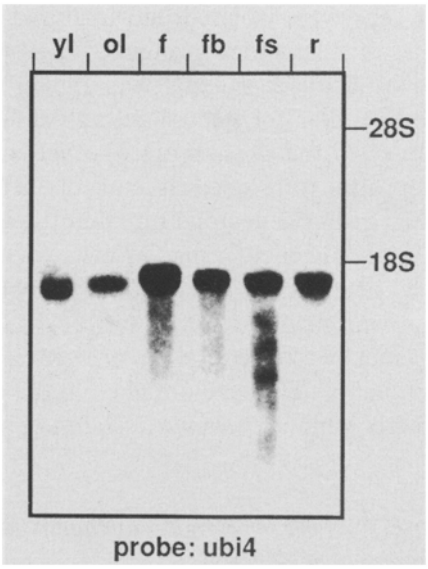

Fig. 3. RNA-blot analysis of parsley ubiquitin mRNA from various organs of a parsley plant. Total RNA (10 μg) from young leaves (yl), old leaves (ol), flowers (f), flower buds (fb), flower stems (fs), and roots (r) of flowering parsley plants was separated electrophoretically, transferred to nylon membrane, hybridized with the ubi4 probe and autoradiographed. The positions of cytoplasmic rRNAs are indicated for size comparison.

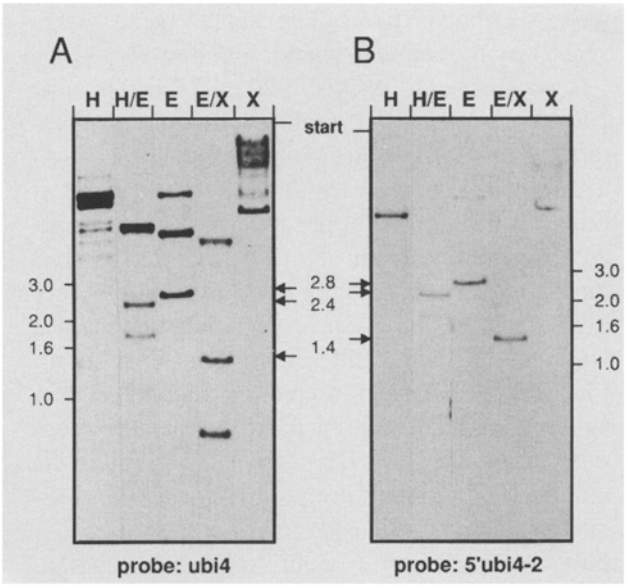

Fig. 4. DNA-blot analysis of parsley genomic ubiquitin genes. Ten μg genomic DNA isolated from suspension-cultured parsley cells was digested with *Eco* RI (E), *Hin*d III (H), *Xho* I (X), or combinations of these enzymes as indicated, size-separated by electrophoresis and transferred to nylon membranes. One filter each was hybridized with the general ubiquitin probe ubi4 (A) and with the *ubi*4–2 specific probe 5′ubi4–2 (B), and both were autoradiographed. Positions of DNA length standards are indicated on the margins. Sizes are given in kb. In the centre, the positions of the 2.8 kb *Eco* RI, the 2.4 kb *Eco* RI/*Hin*d III, and the 1.4 kb *Eco* RI/*Xho* I fragments are given.

Slightly increased hybridization signals were observed in RNA isolated from flowers, and reduced signal intensity was obtained for old leaves.

Polyubiquitin in parsley is encoded by at least two genes

Genomic DNA blots provided initial information on the structure and genomic organisation of the parsley ubiquitin genes. Restriction digests with several different enzymes and hybridization with the ubi4 probe yielded fragmentation patterns of low complexity (Fig. 4A). Additional bands appeared upon longer exposure of the filters. These results suggested that a polyubiquitin gene family exists in parsley. The weaker signals may represent genes encoding ubiquitin extension proteins with only one unit and/or ubiquitin sequences with less similarity to the probe used. Screening of a genomic DNA library [24] with the ubi4 probe led to the isolation of 14 independent clones (gλubi). DNA-blot experiments and restriction analyses of these clones suggested that an internal 2.8 kb *Eco* RI fragment, obtained from gλubi-2, might contain an entire polyubiquitin gene. A fragment of the same size was also detected in an *Eco* RI digest of parsley genomic DNA hybridized with the *ubi*4 probe (Fig. 4A). To further verify the identity of this fragment and to exclude internal deletions, we used a probe derived from a small subfragment of the 2.8 kb fragment for additional DNA-blot experiments. This probe, designated 5′ubi4–2, detected specifically the expected band (Fig. 4B) of about 2.8 kb in *Eco* RI-digested genomic DNA. Double digests with *Eco* RI/*Hin*d III or *Eco* RI/*Xho* I also yielded the fragments expected from the restriction analysis

of gλubi-2. The absence of *Eco* RI sites within this fragment indicated that the encoded gene might be *ubi*4–2.

The 2.8 kb fragment was subcloned and sequenced. The result is shown in Fig. 5. Alignment of the nucleotide sequences of the pubi/14, pubi/15, and pubi/19 cDNAs with the genomic sequence resulted in 100% identity (the various 5′ and 3′ ends of the cDNAs are marked by triangles in Fig. 5). We conclude that the genomic

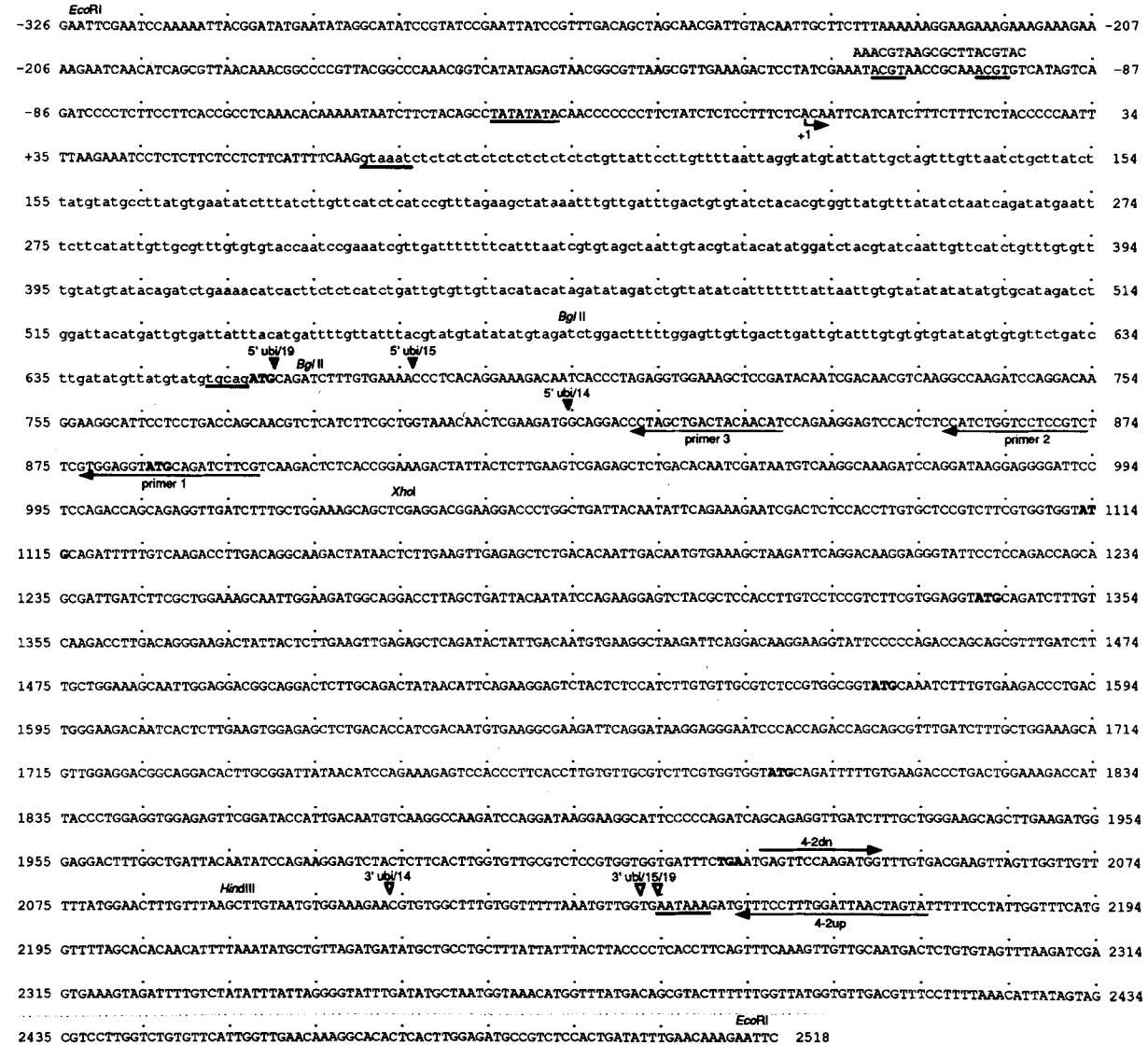

Fig. 5. Nucleotide sequence of the parsley *ubi*4–2 polyubiquitin gene. The 5′ ends (closed triangles) and 3′ ends (open triangles) of the various sequenced *ubi*4–2 cDNAs are indicated. A bent arrow marks the position of transcriptional initiation which is defined as nucleotide position + 1. The positions of the ocs element-like sequence, the putative TATA box, the *Eco* RI, *Xho* I and *Hind* III sites as well as the splice-signal homologies and the assumed polyadenylation signal [29] are marked. For comparison, the original ocs element [8] is shown above the related *ubi*4–2 promoter sequence. Nucleotides given in small characters belong to the intron. Of several *Bgl* II restriction sites, the two used for preparation of the 5′ubi4–2 probe are shown. Arrows indicate the annealing positions of primers used for RACE and for preparation of the *ubi*4–2 specific probe.

2.8 kb *Eco* RI fragment represents the polyubiquitin gene *ubi*4–2.

Determination of the ubi*4–2 transcriptional start site*

Visual inspection as well as computer analysis failed to identify common promoter elements in the vicinity of the protein-coding region of *ubi*4–2. In contrast, a consensus 3′ intron/exon border sequence (5′-Y_{11} TGCAG: G-3′) was detected immediately upstream of the putative translational start codon. Also, no signal was detected on RNA blots hybridized with the 5′ubi4–2 probe which is specific for this region. These data indicated that an intron might be present in this region of the gene. Because the *ubi*4–2 cDNAs did not contain sequences from the 5′ untranslated region, we were unable to deduce the borders of this intron. In addition, it was impossible to design specific oligonucleotides for primer extension experiments to map the transcriptional start site.

These problems were overcome by using a modified version of the PCR-based RACE method. On the basis of sequence comparison of all 12 available parsley ubiquitin-coding repeats, three different primers were designed to allow the specific amplification of primer-extension products spanning the first *ubi*4–2 repeat and the 5′-untranslated region. PCR products of the second amplification reaction were cloned into pKS$^-$ and analyzed for the appearance of restriction sites characteristic of the first *ubi*4–2 repeat. The inserts of 6 plasmids which showed the expected restriction pattern were sequenced and the results compared with the genomic sequence. All six contained sequences ranging from primer 3 into the 5′-untranslated region of the *ubi*4–2 cDNA. Due to differences in the number of T residues at the 5′ end of the cloned PCR products, the inserts differ in size. Nevertheless, all 6 sequenced PRC-amplified primer-extension products terminated at the same position, thereby defining the transcriptional start site (position + 1 in Fig. 5). Sequence comparison revealed the position of a 587 bp intron in the *ubi*4–2 gene (small characters in Fig. 5). The A/T content is 70.2% for the intron, whereas the overall A/T content of the exonic sequences is 53.5%.

The 2.8 kb fragment contains 326 bp of the *ubi*4–2 promoter region. This region includes a TATA box about 30 bp upstream of the transcriptional start site and a sequence reminiscent of the ocs enhancer element around position −110 (Fig. 5).

Parsley polyubiquitin genes encode hexameric precursor proteins

From the nucleotide and deduced amino acid sequences of the *ubi*4–1 cDNA and from the coding region of the *ubi*4–2 gene we draw the following conclusions: (1) both genes contain six repeat units, each coding for one ubiquitin monomer; hence, *ubi*4–1 and *ubi*4–2 are hexaubiquitin genes; (2) the C-terminal monomers are followed by the same two amino acids, aspartic acid and phenylalanine; (3) all monomers from both genes are identical in amino acid sequence; (4) no intron disrupts the *ubi*4–2 coding sequence; (5) the *ubi*4–1 cDNA contains a putative polyadenylation signal 23 nucleotides upstream of the beginning of the poly(A) tail. This signal is also present in *ubi*4–2 at a similar position.

Specific probes for ubi*4–1 and* ubi*4–2 detect constant mRNA levels*

Probes derived from the 3′-noncoding sequences of *ubi*4–1 and *ubi*4–2 were used to assay for the expression of the two genes in a gene-specific manner. Specific hybridization was observed with the two types of polyubiquitin cDNA under stringent conditions. In addition to the response to heat shock, we tested the effects of UV-containing white light and of a fungal elicitor on possible differential activation of the two polyubiquitin genes. The results obtained with the two gene-specific probes (Fig. 6) indicate more or less constitutive expression, similar to earlier studies

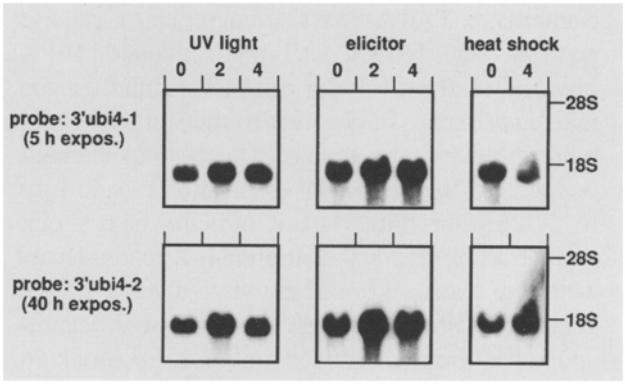

Fig. 6. RNA-blot analysis of *ubi*4–1 and *ubi*4–2 epxression in response to various stimuli. RNA prepared from cultured parsley cells, which had been subjected to the treatments indicated for the time periods given in hours, was size-separated electrophoretically and transferred to nylon membrane. Two identical filters were hybridized either with the 3′ubi4–1 probe (upper part) or with the 3′ubi4–2 probe (lower part) and autoradiographed for the time indicated. The positions of cytoplasmic rRNAs are indicated for size comparison.

with the ubi4 probe [32–34, 54, 56]. Different signal intensities, reflected by different exposure times of the two autoradiograms, suggest that *ubi*4–1 contributes about eight times more to the total polyubiquitin mRNA level than *ubi*4–2 does.

Discussion

Our results demonstrate the existence of at least two hexaubiquitin genes in parsley. One of them, Pc*ubi*4–1, was analysed in the form of one near full-length and several partial cDNAs, the other, Pc*ubi*4–2, in the form of cDNAs and cloned genomic DNA. The existence of cDNAs with identical sequences and gene-specific probing clearly shows that the genomic 2.8 kb *Eco* RI fragment encodes an active polyubiquitin gene. Expression studies indicate that a variety of parsley tissues and cultured parsley cells contained high levels of polyubiquitin-encoding (*ubi*4) mRNAs. No appreciable changes in expression occurred in response to light treatment, elicitor treatment or heat shock, neither with the ubi4 probe nor with gene-specific probes for the two types of polyubiquitin mRNA analysed.

The unequivocal identification of CON2 as an ubiquitin-encoding gene was facilitated by the exceptionally high degree of amino acid sequence conservation among ubiquitin proteins. Even the ubiquitin portion of a ubiquitin extension protein from a lower plant (*Chlamydomonas reinhardtii*) differs only at amino acid position 24 from the higher plant ubiquitins [39]. The deduced amino acid sequence for ubiquitin in parsley was identical with that reported for ubiquitins from other higher plants [40], including *Arabidopsis* [10], sunflower [5] and the monocotyledonous plant maize [12]. It is noteworthy that the C-terminal extensions of polyubiquitins encoded by the *Arabidopsis* and both parsley *ubi*4 genes consist of two (non-identical) amino acids, in contrast to other known cases with only one additional amino acid [19, 40]. Due to differences in 'wobble' positions, the nucleotide sequences encoding the individual repeat units are only about 84% identical within each gene as well as between the two genes. Values in the same range result from any combination of repeat units from the *Arabidopsis ubq*4 and the two parsley *ubi*4 genes. The number of repeats is also very similar for the polyubiquitin genes in parsley [6], *Arabidopsis* [5], maize [7], and sunflower [6].

We have shown that the overall transcription rate of parsley *ubi* genes is not affected by continuous heat shock, although different heat-shock conditions may lead to different results (see [12]). Earlier results obtained for light or elicitor treatment of culture parsley cells [32–34, 54, 56] indicated that ubiquitin mRNA levels and gene activity do not significantly change in response to these stimuli. In these earlier studies, the ubi4 (con2) probe was used, which most probably detects all ubiquitin sequences. Here we show that *ubi*4–1 and *ubi*4–2 mRNA levels are not differentially influenced and remain largely constant after application of the respective stimulus. This suggests that both genes are neither induced nor repressed by the stimuli tested. However, due to the small size of the gene-specific probes, expres-

sion at the level of gene activity was not measured.

It is interesting to note that the ubi4 probe detects only one size class of mRNA in RNA-blot experiments. Similar results were obtained with RNA from cultured cells as well as intact parsley plants, regardless of whether total or poly(A)$^+$ RNA was analysed. In contrast, the parsley ubi4 probe detects 4 hybridizing bands with RNA from *Arabidopsis* [53]. This pattern is similar to that obtained by Burke *et al.* [10] with a homologous ubiquitin probe. One explanation is that in parsley, in contrast to other systems, all ubiquitin-encoding mRNAs are of the same size. Further studies, especially with respect to ubiquitin extension proteins, are necessary to clarify this possible difference between parsley and other plants, such as *Arabidopsis*, sunflower [6], tomato [25], maize [12] and barley [21], all of which express several size classes of ubiquitin mRNAs.

The ubi4–2 transcriptional start site was determined by a modification of the RACE protocol [20]. Sequence analysis of the resulting 6 cloned PCR-amplified primer-extension products, and comparison with the genomic sequence defined the transcriptional start site and the location of an intron. The 587 bp intron is located immediately 5′ to the translational start codon, contains at its ends the exon/intron-junction consensus sequences (5′-AG:GTAAGT-3′, 5′-Y_{11}TGCAG:G-3′ [9]), and shows an increased A/T content which is a common feature of plant introns [57]. An intron was also shown to be present at a very similar position in two sunflower (Ha*ubb*1 and 2 [6]) and in two maize (Zm*ubi*1 and 2 [13]) polyubiquitin genes, and a closely related gene organization has been reported for human and chicken polyubiquitin genes [3, 7]. In the maize *ubi*1 and *ubi*2 genes, it has been shown that the intron is inefficiently spliced upon a severe heat shock. The question of whether this conserved intron is involved in the regulation of expression of polyubiquitin genes in plants was addressed in the sunflower system. By transient expression analysis, no effect of the intron on gene expression was detected [4].

With respect to possible regulatory promoter elements, a TATA-box sequence occurs around position −30. No CCAAT-box sequence [16] is obvious in the *ubi*4–2 promoter similar to the maize *ubi*1 gene. In contrast to the sunflower and maize polyubiquitin genes, no heat-shock element is detected in the P*cubi*4–2 promoter region up to −326. Our data do not indicate heat-shock regulation of the *ubi*4–1 and *ubi*4–2 genes. However, the transcriptional activity of most of the genes not directly related to the heat-shock response is greatly reduced under heat shock in various systems [35], and this also applies to *pr*1 and several other elicitor-responsive genes in parsley (I. Somssich, unpublished results). It is interesting to note that ubiquitin gene expression seems to be hardly affected by heat shock in parsley cells. Further experiments are required to determine whether this is due to compensatory effects, or to a very brief, transient induction of the *ubi*4–1 and *ubi*4–2 mRNAs, which was not detected in the RNA-blot experiment using the gene-specific probes.

A promoter element of possible functional relevance for *ubi*4–2 gene expression might be the sequence resembling an ACGT element (ACE) [1, 56] around position −110; which is similar to the ocs [8] and the as-1 [31] elements. These elements, often containing twice the sequence ACGT separated by eight base pairs, are well characterized in plant pathogen promoters (e.g. as-1 in the 35S promoter of the cauliflower mosaic virus), and can be bound by two dimers of plant bZIP DNA-binding proteins [1, 48]. Interestingly, a sequence similar to the G-box [22] which also contains the ACGT core is present in a short promoter region found to be important for activity of the sunflower *ubb*1 gene [4]. From the sequence similarity with other ACEs, it can be predicted that the element within the *ubi*4–2 promoter will be recognized by plant bZIP proteins present in parsley nuclear extracts (see [1]). Sequence similarity and possible protein binding might suggests a role of the ACE sequence in *ubi*4–2 gene regulation, a hypothesis which will be tested by promoter analysis in parsley protoplasts.

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