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# Isolation and characterization of tomato cDNA and genomic clones encoding the ubiquitin gene *ubi3*

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## Abstract

We report here the isolation and nucleotide sequence of tomato cDNA and genomic clones encoding a ubiquitin extension protein homologous to the yeast gene *ubi3*. Sites similar to upstream activating sites commonly found in the promoters of yeast ribosomal genes were observed in the tomato promoter. The tomato *ubi3* promoter also contained elements found in the *rbcS* promoter from pea. The transcription initiation site was determined to occur 66 bp upstream of the initiating Met. RFLP mapping revealed that the gene was located on chromosome 1, 23 cM from marker TG301. A *ubi3* gene-specific probe hybridized to a single 800 nt transcript. Expression was reduced in heat-shocked plants and plants kept in the dark. Expression was highest in young leaves and immature green fruit and lowest in mature leaves and petals. We isolated the original cDNA clone using an antibody prepared against chloroplast polypeptides. Immunological studies did not detect ubiquitin or ubiquitin extension proteins in the chloroplast. However, higher-molecular-weight chloroplast proteins were detected with ubiquitin antisera suggesting that ubiquitin conjugates are transported into the chloroplast.

## Introduction

Ubiquitin is a highly conserved 76-residue protein found in all eukaryotic cells. It is found free or covalently joined, through its carboxyl terminal glycine residue, to the ε-amino group of a lysine residue in various cytoplasmic, nuclear, and integral membrane proteins [6]. Proteins conjugated to multiubiquitin chains are selectively degraded while proteins conjugated to monoubiquitin are often stabilized [6].

In yeast, four ubiquitin genes have been isolated [26] all of which encode ubiquitin polyproteins. The yeast *ubi4* gene encodes 5 copies of

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X58253.

ubiquitin tandemly linked whereas *ubi1*, *ubi2*, and *ubi3* encode polyproteins consisting of a single copy of ubiquitin fused to highly basic, cysteine-rich polypeptides. The yeast *ubi1* and *ubi2* genes encode ubiquitin fused to a 52 amino acid C terminal extension, whereas *ubi3* encodes a 76 amino acid extension with little sequence similarity to the extensions of the UBI1 and UBI2 proteins. Recently it was demonstrated that the 52 and 76 amino acid extensions of UBI1, UBI2, and UBI3 are polypeptide components of the 80S ribosomes [9, 23, 30]. Furthermore, the incorporation of the extensions into ribosomes was promoted by their association with ubiquitin, leading Finley *et al.* [9] to suggest a novel chaperone function for ubiquitin. Ubiquitin has also been found to promote the translocation of monoamine oxidase B into the mitochondrial outer membrane [37], a function characteristic of other chaperone-like proteins [7, 25, 29].

In plants, ubiquitin is known to play a role in the degradation of the Pfr form of phytochrome [32]. Plant ubiquitin genes homologous to *ubi1* and *ubi2* [3, 13], *ubi3* [4, 12] and *ubi4* [2] have recently been cloned and sequenced. Callis *et al.* [4] have also provided evidence that the extension proteins are associated with cytoplasmic ribosomes.

In this report we describe the cloning of a ubiquitin gene highly homologous to yeast *ubi3* that we isolated while screening a tomato cDNA library with antibodies made against chloroplast polypeptides from photosystem I. Unlike the yeast gene, the cDNA clone we isolated contained no stop codons upstream of the first methionine suggesting the possibility that plants contain a form of UBI3 that has a transit peptide and is targeted to the chloroplast. The recent report that *Chlamydomonas* chloroplasts contain protein that binds to ubiquitin antibody is also consistent with this notion [36]. To examine this possibility we further isolated and characterized a tomato *ubi3* genomic clone. We present the gene sequence, its chromosomal location, transcription initiation site, and data on its expression during development and under stress. Our results indicate that the UBI3 protein does not contain a transit peptide. Furthermore, using ubiquitin antibodies as probes, we were unable to detect any free ubiquitin in the chloroplasts of spinach or pea. These antibodies, however, recognized several higher-molecular-weight chloroplast proteins in both pea and spinach.

## Materials and methods

### *Library screening*

The construction of the tomato cDNA expression library and procedures employed for screening the library are described and referenced in Hoffman *et al.* [17]. The polyclonal antiserum used in the identification of cDNA clones was prepared against *Vicia faba* 14–20 kDa photosystem I polypeptides. Briefly, photosystem I polypeptides were prepared according to Mullet *et al.* [24], electrophoresed on preparative denaturing SDS-PAGE gels, and polypeptides in the 14–20 kDa range were electroeluted from the gel and used as antigen in New Zealand rabbits. The tomato genomic library was a gift from Dr. W. Gruissem, Dept of Botany, University of California at Berkeley, CA [34]. The genomic library was plated using the *Escherichia coli* host Q358 and duplicate filters were screened using a 574 bp *Bgl* II-*Sty* I subfragment or a 282 bp *Alu* I-*Sty* I subfragment of the *ubi3* cDNA insert as radiolabelled probes as outlined in Maniatis *et al.* [22]. The *Bgl* II-*Sty* I probe hybridizes to all ubiquitin genes while the *Alu* I-*Sty* I probe is specific for *ubi3*. Filters were prehybridized for 10 min in 6 × SSC containing 0.25% non-fat milk and hybridized in the same buffer containing $10^6$ cpm/ml probe at 68 °C for the large probe and 50 °C for the small probe. Filters were washed in 0.1 × SSC containing 0.1% SDS at room temperature. Colonies detected on duplicate filters were plaque-purified and subcloned for further analysis.

The nucleotide sequence of the *ubi3* cDNA and genomic clones were determined by the dideoxynucleotide chain termination method on double-stranded DNA templates [31].

*Expression studies and primer extension*

Total plant RNA samples used for analyzing tissue-specific expression were isolated from field-grown tomato (*Lycoperiscon esculentum* cv. Big Girl; Burpee Seed Co). Leaf tissue was from either the youngest (first leaf) or fourth leaf from the meristem. Petioles were from the fourth leaf. Only fully open flowers were harvested. One collection comprised yellow flower petals only and the other contained a mixture of petals and ovaries. Fruit were designated stage 1 when green with immature seeds, stage 2 when green but seeds were fully developed, stage 3 when color was first evident, and stage 4 when fully red. Tissue was harvested between 13.00 and 14.00, frozen in liquid nitrogen, and stored at −80 °C until extracted.

Heat or light/dark treatments were given to seedlings grown in growth chambers under cool fluorescent light (14 h light/10 h dark), at 22 °C. Plants were used when they had two sets of true leaves. Dark treatments were administered by maintaining light-grown plants in a darkened growth chamber for 3 days. For heat shock treatments plants were moved from the illuminated chamber into a 42 °C incubator for 1 hour. RNA samples were isolated as described [5]. Total RNA was separated from DNA by precipitation with 2 M LiCl. All RNA samples were separated on formaldehyde gels, transferred onto nitrocellulose filters and hybridized according to Maniatis *et al.* [22] using the ubiquitin or *ubi3*-specific probes employed for genomic clone isolation. Negatives of the ethidium-stained RNA gels and autoradiographs of northern blots were scanned using an LKB ultrascan XL laser densitometer.

Primer extension was done as described [18] using the oligo 5′-CTTCGTCTGGAGGAGAG-3′.

*Immunological blotting*

Antibodies prepared against ubiquitin and the UBI3 tail and their respective pre-immune sera were provided by Judy Callis, Dept of Biochemistry, Univ. of California, Davis [4]. Ubiquitin antibodies were also purchased from Sigma. After SDS-PAGE, proteins were transferred to nitrocellulose and the blots were autoclaved for 20 min in transfer buffer (25 mM Tris, 193 mM glycine pH 8.3). Blots were blocked in TBS (10 mM Tris pH 7.5, 150 mM NaCl) containing 2% non-fat dry milk. Antibodies were diluted 1:200 in a solution of TBS, 2% milk, 0.05% NP40, and 1% Triton X-100. Blots were shaken in the antibody solution overnight at 4 °C. After washing successively in TBS, a solution comprised of TBS + 1% Triton X-100 + 0.05% NP40, and TBS, blots were incubated for 1 h at room temperature in Protein A-alkaline phosphatase (Sigma) diluted 1:2000 in TBS containing 2% non-fat milk. Blots were washed in TBS, TBS + 0.05% NP40, and AP 9.5 (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Blots were developed in AP 9.5 containing 0.33 mg/ml Nitroblue tetrazolium and 0.17 mg/ml bromochloroindolyl phosphate-toluidine salt.

## Results

*cDNA clone selection and identification*

Antibodies prepared against *Vicia faba* 14–20 kDa photosystem I polypeptides were initially used to screen a tomato cDNA expression library in an attempt to isolate genes encoding photosystem I polypeptides. This antiserum cross-reacted with several tomato polypeptides primarily in the 14–20 kDa range (Fig. 1). We isolated a clone containing a 685 bp insert having an ORF of 178 residues and theoretical molecular mass of 17.7 kDa. The predicted polypeptide was very basic with an estimated pI of 9.9. A comparison of this sequence to entries in the GenBank revealed that it was related to the ubiquitin extension protein, UBI3, cloned from yeast [26], and homologues in *Drosophila* [20], man [21], *Arabidopsis* [4] and barley [12]. The UBI3 homologues from all six species are highly conserved (Fig. 2). All the peptides are predicted to contain a 76 amino acid ubiquitin polypeptide fused to a basic

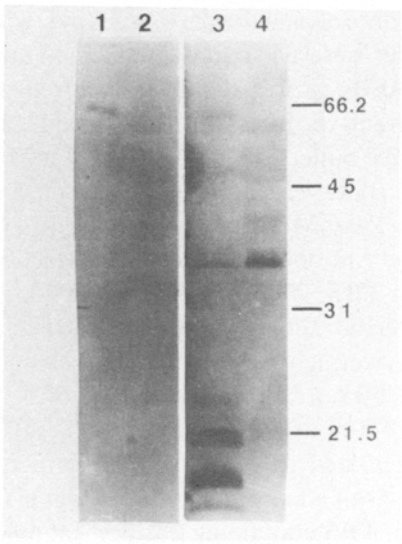


*Fig. 1.* Immunoblot analysis of tomato chloroplast proteins with antisera against UBI3 and PSI. Thylakoids and stroma were prepared from intact chloroplasts that were hypotonically lysed. Each lane was loaded with sample prepared from chloroplasts containing 20 $\mu$g of chlorophyll. Lanes 1, 3: thylakoids; lanes 2, 4: stroma. Lanes 1 and 2 were probed with the antibody specific for UBI3. Lanes 3 and 4 were probed with antisera against PSI. Molecular weight markers (BioRad), in kDa, are indicated to the right of the figure.

C terminal extension. This extension is predicted to be 80 residues for tomato, *Drosophila*, and humans but is 76 residues in yeast, 79 residues in barley, and 81 residues in *Arabidopsis*. The predicted ubiquitin amino acid sequence is identical between tomato, barley, and *Arabidopsis* and is over 96% identical between the plant and non-plant species. The extension portion is 65–75% identical between the non-plant species but is 88% identical between tomato and *Arabidopsis* and over 92% identical between tomato and barley (Fig. 2). Yeast and *Drosophila ubi3* genes encode an in-frame stop codon 21 and 15 bp upstream of the initiating methionine. In contrast, the tomato cDNA contained at least 66 bp of open reading frame (Fig. 3A). Since we had isolated the tomato-ubiquitin-cDNA clone with an antibody prepared against chloroplast proteins, we tested the hypothesis that the upstream open reading frame of the tomato clone encodes a transit peptide that directs the ubiquitin polyprotein to the chloroplast and that we had isolated an incomplete cDNA. Attempts to isolate a cDNA clone containing additional 5′ sequence were unsuccessful, however.

### *Isolation of* ubi3 *genomic clone*

To further pursue whether the upstream open reading frame encoded a genuine transit peptide, we used the cDNA clone to isolate a tomato *ubi3* genomic clone. From approximately $2.5 \times 10^5$

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          10         20         30         40         50         60         70
T:MQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLADYNIQKESTLHLV
A:------------------------------------------------------------------------
B:------------------------------------------------------------------------
D:------------------P----E-------------------------------S-------------
H:------------------P----E-------------------------------S-------------
Y:--------------------------S-----------------------------S-------------
        ↓ 80         90         100        110        120        130        140
T:LRLRGGAKKRKKKTYTKPKKIKHKKKKVKLAVLQFYKVDD.TGKVQRLRKECPNAECGAGTFMANHFDRH
A:-----------------------H--------------G.S-------------T--------S------
B:--------------------Q---H---------------A----T---------D--------------
D:-------------N-ST-------R---------KY----E.N--IH---R---GEN----V---A-E---
H:-------------S--T---N---R---------KY----E.N---S---R---SD-----V---S-----
Y:------G------V--T-------H---------SY----A.E---TK--R--S-PT----V-L---K--L
         150
T:YCGKCGLTYVYN.KAGGD
A:-----------Q.-E-AQE
B:------------Q--
D:-----N--F-FS.-PEEK
H:-----C---CF-.-PEDK
Y:-----HSV-KV-.A
```

*Fig. 2.* Comparison of the predicted protein sequences of UBI3 and homologues. T, tomato; A, *Arabidopsis* (*ubq6*) [4]; B, barley (*mub1*) [12]; D, *Drosophila* [20]; H, human [21]; Y, yeast [26]. The first amino acid of the extension polypeptide is marked with an arrow.

```
   1 GATATCTTTT TGTCATGATA TAATATGTTT CTTTTAGCAA AAATGAAATA GTTCCGTCAT TTATCGGATC ATATTTAGAT AGATTGTTGT AGATGACACT TTTATGAAAT
 111 TACAATAATC ATAAATTTAT CACGGGCACA ATTAGCGACA ATGAAGACCA ACCAAAGTTA GGGGGCAAAA ACCGAACAGG CCAACCAAAG TTATGGGTGT GCAAAAAATC
 221 GAATCGATCG ATAAATCGAA TCGAAAAAAT GTTATTGGGT TATTTATGTT TTTTCGTGGG TTTATAAAAA AAATTATTGA ATTATTGGTT CGGTTTCAAT TTTTATTATT
 331 GGGTTATTGG GTAAACCGAT AATCCAATAA GACGGTAATA ATTTTTATTT TACCCTTCAT AATTATTTAT TATTAGCAAG TTAATATATA ATTAGACACT ATAATTATAT
 441 CAAATTATTA GTACTCTACC AACTTCAGAG TTGGCTGATT TACTAGTTTT TATTGTTTAT TCAAAACCTA AGAATTAAAG TAATGCGTTC ACAATTGCAG TTATTTGATT
 551 TTAATTTTAG TTTTAGTCTT ATTGGACTGT TTTATTTTAG TTTTAGGTTA TAATGGCAGG TTATAGCATT TGCATCTTAG TAAAGGTCAG TAATTTGATT AACACAAAAA
 661 ATTCCATACT ATATTTTGGT GGTAATATGT AATTATAGCA TTCGTGCTAT TTTCTCGTAT TGATACAATT TCTCATTATC TTTCTTGTTT TACTATATCA AAACATTTAG
 771 AGAAGTGAGA AGACATATAA TATTTTACGG ACATTTTCTT ATTGGGTAAA CTGAAAATCG AATCGATAAT GATAAAAAAC TGATAAATTG AAATCTGATA AAAAATATCT
 881 TGTTGATTTG TTATTGAATT AACATATTTA AAAGCTGAAA ACCGATAAAT CGAACGATAA TATATAAAAT CGAACCGAAC CGATCGATGC ACACCCTTGA CCAAATCTGA
 991 AGCACATATT TATCGATCTA AATTTTATTA AAGAGATTAA TATCGAATAA TCATATACAT ATTTCATATG TATAACAAAT TTCAAATACA CGTATCTAAT ATATCGAGTG
1101 ATGCGACAAA TACATGTATC GGACGCACCA ATTGATATAG AAAACGTAAT ATTGAAAACT AATGTAAAGA AAAGTAACTT GATCCTAAAC TAATCAAGAT AAGCCCAATA
1211 AATATACATT GTCATCTCCA AAGGCCCAAA AATGGCACAA GATGGCAGGC CCAATAACGA AGAAAAGGGC TTGTAAAACC CTAA TAA AGT GGC ACT GGC AGA GCT
                                                                                                  *  S  G  T  G  R  A>
1316 TAC ACT TTC ATT CCA TCA ACA AAA GAA ACC CTA GAA GCC GCA GTG CCA CTG ATT TCT CTC CTC CAG ACG AAG ATG CAG ATC TTC GTG AAA
     Y  T  F  I  P  S  T  K  E  T  L  E  A  A  V  P  L  I  S  L  L  Q  T  K  M  Q  I  F  V  K>
1406 ACC CTA ACG GGG AAG ACG ATC ACC CTA GAG GTT GAG TCT TCC GAC ACC ATC GAC AAT GTG AAA GCC AAG ATC CAG GAC AAG GAA GGG ATT
     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>
1496 CCC CCA GAC CAG CAG CGT TTG ATT TTC GCC GGA AAG CAG CTT GAG GAT GGT CGT ACT CTT GCC GAC TAC AAC ATC CAG AAG GAG TCC ACT
     P  P  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>
1586 CTC CAT CTC GTG CTC CGT CTC CGT GGT GGT GCT AAG AAG AGG AAG AAG AAG ACC TAC ACC AAG CCA AAG AAG ATC AAG CAC AAG AAG AAG
     L  H  L  V  L  R  L  R  G  G  A  K  K  R  K  K  K  T  Y  T  K  P  K  K  I  K  H  K  K  K>
1676 AAG GTT AAG CTC GCT GTG TTG CAG TTC TAT AAG GTT GAT GAC ACT GGA AAG GTT CAG AGG CTT CGT AAG GAG TGC CCT AAT GCT GAG TGC
     K  V  K  L  A  V  L  Q  F  Y  K  V  D  D  T  G  K  V  Q  R  L  R  K  E  C  P  N  A  E  C>
1766 GGT GCT GGA ACT TTT ATG GCT AAC CAT TTT GAC CGT CAC TAC TGT GGT AAG TGT GGG CTC ACC TAC GTT TAC AAC AAG GCT GGA GGC GAT
     G  A  G  T  F  M  A  N  H  F  D  R  H  Y  C  G  K  C  G  L  T  Y  V  Y  N  K  A  G  G  D>
1856 TGATT TTAATGTTTA GCAATGCTCT ATCAGATTTT CTTTTTGTCG AATGAACGGT AATTTAGAGT TTTTTTTTTG CTATATGGAT TTTCGATTTT GATGTATGTG
1961 ACAACCCTTG GGATTGTTGA TTTATTTCAA AACTAAGAGT TTTTGCTTTA ATGTTCTCGT CTATTTTCGA TATCAATCTT AGTTTTATCT CATTCTAGTT GTCTAATGTT
2071 CAACATATTA GCAATTTGGC GGATTATAGA ACTATCAAAT ATGCTTCTCA GGAAATTTGA GATTTACCAG TCCTTGTGCT CATGGGGTTG AGTATAATAT AGGAAAAAAT
2181 AGTAAATTTA AGCCTGTGCT ATGTTTCTAT ACTTTTATTT ATTTGTCTCT GTACTTCCTC ATGCTGAAAC TCTGCTGTGC ATTTCATTAA TTTGAGAAAC ATAAATAAAG
2291 GGAACTGAGA AGGGACTGCC TGTTTGGTTG TGTGTGCTAC ATTTAGTAAT TCTGTAGTAT AGATTGCATT ATATGCTTTT AGCG
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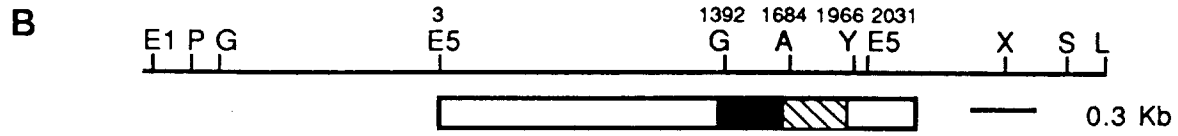


*Fig. 3.* DNA sequence and map of the tomato *ubi3* genomic clone. A. The sequence is numbered from the 5′ *Eco* RV site. The *ubi3* cDNA clone begins at residue 1324 and extends to residue 2011; these nucleotides are marked with a dot. The predicted protein sequence is shown extending to the first stop codon 5′ to the initiating methionine (boxed). The major transcription initiation site identified by primer extension is indicated with a bent arrow. Putative TATA and CAAT boxes are underscored with dashed lines. The sequence complementary to the oligo used for primer extension begins at residue 1371 and extends to residue 1387. Direct repeats and the repetitive element TTATTGGG are underlined. B. Restriction map of the *Eco* RI-*Sal* I genomic fragment containing the *ubi3* gene. The boxed region was sequenced. The darkened area represents the ubiquitin sequence and the hatched area represents the UBI3 tail. Restriction sites are indicated as follows: E1, *Eco* RI; P, *Pst*; G, *Bgl* II; E5, *Eco* RV; A, *Alu* I; Y, *Sty* I; X, *Xba*; S, *Sac* I; L, *Sal* I. Numbers refer to the cleavage site corresponding to the sequence in A.

phage plaques we isolated six putative ubiquitin genomic clones; only one hybridized very strongly to the *ubi3*-specific probe (the *Alu* I-*Sty* I subfragment, see Fig. 3b). The other clones are likely to include other members of the ubiquitin gene family [2]. The cDNA probe hybridized to a single 5 kb *Sal* I-*Eco* RI fragment prepared from the strongly hybridizing genomic clone. This subfragment was subcloned into pBLUESCRIPT for restriction enzyme mapping and DNA sequence analysis. The restriction map is shown in Fig. 3B. The nucleotide sequence of the *ubi3* gene and flanking regions are shown in Fig. 3A. The genomic clone is confirmed to encode the cDNA since the two sequences are identical within the corresponding regions (Fig. 3A). The upstream

open reading frame only continues for an additional ten amino acids before reaching a stop codon. As no methionine residues are found in this region it is highly unlikely that this sequence encodes a transit peptide.

*Structural features of the genomic clone*

From the sequence analysis, we observed that tomato *ubi3*, like the homologous yeast, *Arabidopsis* and barley genes, contains no introns within the coding region. Using primer extension, we mapped the transcription initiation site in RNA prepared from leaf tissue. The major primer-extended product corresponded to nucleotide 1322. Two additional minor products corresponding to nucleotide 1321 and 1327 were also observed (data not shown). Putative TATA and CAAT boxes are located 30 and 60 nt, respectively, upstream of the major transcription initiation site. The major transcription initiation site is only two base pairs upstream of the longest *ubi3* cDNA we isolated. All three sites of transcription initiation are consistent with translation initiation at methionine codon beginning with nucleotide 1388.

Most yeast genes encoding ribosomal proteins contain common upstream elements, termed upstream activating sites (UAS), that are located 250–450 nucleotides upstream of the AUG start codon and that promote transcription [28]. The yeast consensus UAS sequence is shown in Fig. 4 aligned to similar sequences found in the tomato *ubi3* promoter. We note that the tomato *ubi3* gene contains six putative UAS elements located 280–1300 nt upstream of the AUG start codon. Gausing and Jensen also reported that the promoter of the homologous barley genes contain putative UAS elements [12].

We observed that the tomato *ubi3* gene contains sequences similar to footprinted regions of the *rbcS* promoter. These include the L box [14], and box I [19], box II [16], and AT-1 [8] (Fig. 4). In addition to the putative functional elements similar to those found in other promoters, the first 800 bp of sequence contain repetitive DNA of unknown significance. Some of these repeats are indicated in Fig. 3A and include the motif TTATTGGG which appears 5 times and TTTTAG which appears four times.

*Chromosome mapping of* ubi3

RFLP mapping was carried out with a segregating F2 population of the interspecific cross *Lycopersicon esculentum* × *L. pennellii* which is polymorphic for numerous markers, as previously described [1]. A 298 bp *Taq* I-*Bgl* II fragment, derived from the promoter region just upstream

```
TOM ubi3 114-128        AATAATCATAAATTT
TOM ubi3 211-197        CACACCCATAACTTT
TOM ubi3 659-673        AAATTCCATACTATA
TOM ubi3 913-932        AAAACCGATAAATCG
TOM ubi3 1108-1121      AA-ATACATGTATCG
CONSENSUS TOM ubi3      AAAAYCCATAAATYK

CONSENSUS UAS:          AACAYCCRTRCATYW (Planta and Raue, 1988)
CONSENSUS TOM ubi3      **A*******A***K

L box                   AAATTAACCAAC (Giuliano et al., 1988)
TOM ubi3 481-470        ****A*T*****

GT-1 box                GTGGTTAATATG (REF (Green et al., 1988)
TOM ubi3 679-689        *****-******

box 1                   TTTCAAA (Kuhlemeier et al., 1988)
TOM ubi3 1070-1077      *******

AT-1 box                AATTATTTTTATT (Datta and Cashmore, 1989)
TOM ubi3 367-384        ***A*********
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*Fig. 4.* Putative promoter elements found in the 5′ flanking region of tomato *ubi3*. Y = C or T; W = A or T; K = G or T; R = A or G.

of the coding region, was used as a probe and it hybridized to a single fragment in the tomato genome. The F2 segregation analysis (data not shown) indicated that this fragment maps to the end of chromosome 1, 23 cM from marker TG301 (S.D. Tanksley, unpublished).

*Expression of* ubi3

To observe whether *ubi3* expression was suggestive of a role for UBI3 other than in protein synthesis, the expression characteristics of *ubi3* were examined using northern hybridization analysis of RNA samples extracted from various plant organs as well as young tomato leaves subjected to heat or light/dark treatments (see Materials and methods and legend for details). The cDNA hybridization probes utilized were from DNA sequences specific to the ubiquitin-coding region, to the nucleotides encoding the *ubi3* tail, or to the small subunit of ribulose bisphosphate carboxylase, *rbcS*. The resulting autoradiograms are shown in Figs. 5 and 6. The general ubiquitin-specific probe hybridized to transcripts of 1600 and 800 nt. The 1600 nt species hybridized several-fold more strongly than did the 800 nt species. The *ubi3*-specific probe hybridized to a single 800 nt species. Both ubiquitin transcripts were several-fold less abundant than the *rbcS* transcript.

Analysis of total RNA prepared from plants given light and dark treatments suggests that the steady-state levels of *ubi3* mRNA is regulated by light. The *ubi3* transcript present in light-grown tomato plants was reduced 75% in light-grown plants after a 3-day continuous dark treatment (Fig. 5, lane 3A vs. 4A). The light activation observed for this gene might be modulated by the putative light regulatory elements found upstream in the genomic DNA sequence discussed in the previous section. A one-hour 42 °C heat shock also reduces the steady-state *ubi3* level (Fig. 5, lane 1A vs. 2A). In contrast, the level of the 1600 nt species was slightly increased by dark treatment (Fig. 5, lane 3B vs. 4B) but also decreased in response to 42 °C heat shock treatment (Fig. 5, lane 1B vs. 2B). The same blot used

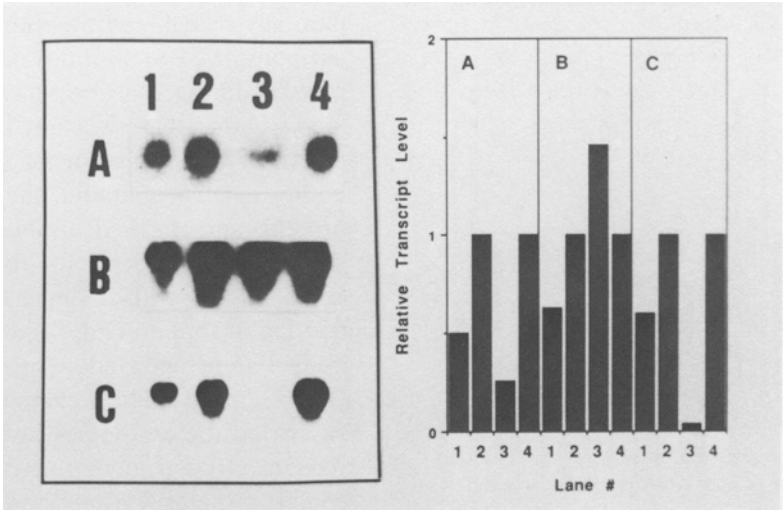


*Fig. 5.* RNA blot analysis of *ubi3* transcript levels in response to light/dark and temperature treatments. Each lane contains 8 μg of total RNA prepared from tomato plants grown in the light and subjected to 1 hour of 42 °C (lane 1), 1 h of room temperature (lane 2), 3 days of darkness (lane 3), or 3 additional days of light. A. The blot was hybridized with the *ubi3*-specific probe and exposed against film for 7 days (only the 800 nt area is shown). B. The same blot in A was stripped of probe, hybridized to the general ubiquitin probe, and exposed for 4 days (only the 1600 nt area is shown). C. The same blot in B was stripped of probe and hybridized to the *rbcS* probe and exposed for 1 day. Relative transcript level is the ratio of the hybridization signal to the signal of the ethidium-stained 16 S RNA band. The relative transcript level of the control sample in lane 2 was arbitrarily set at 1.

in Fig. 5A and 5B was hybridized to the *rbcS* probe for comparison (Fig. 5C). Dark treatment resulted in barely detectable levels of *rbcS* transcript and heat shock also reduced the steady-state level.

The steady-state level of *ubi3* transcript clearly exhibits an organ-specific pattern. This RNA species was most abundant in young leaves and green immature fruits (Fig. 6A, lanes 11 and 4, respectively). The transcript was also highly expressed in samples from other stages of fruit development, stems, ovaries, pedicels, and green sepals (Fig. 6A, lanes 1, 2, 3, 5, 8, 10 and 12, respectively). It was lowest in the yellow flower parts and mature leaves and petioles (Fig. 6A, lanes 6, 7 and 9). In contrast, the 1600 nt transcript, hybridizing to the general ubiquitin probe, was strongly and uniformly expressed in every organ tissue examined (Fig. 6B, lanes 1–12). For comparison, the blot was also hybridized to tomato *rbcS*. *RbcS* transcripts were abundant in vegetative photosynthetic tissue (Fig. 6C, lanes 5, 6, 9–12) including mature leaves that lacked appreciable amounts of the *ubi3* transcript (Fig. 6C, lane 9). Young green fruits and ovaries contained lesser amounts of *rbcS* transcript (Fig. 6C, lanes 2, 4 and 8) and the level was low to undetectable in flowers and ripe fruit (Fig. 6C, lanes 7, 3 and 1).

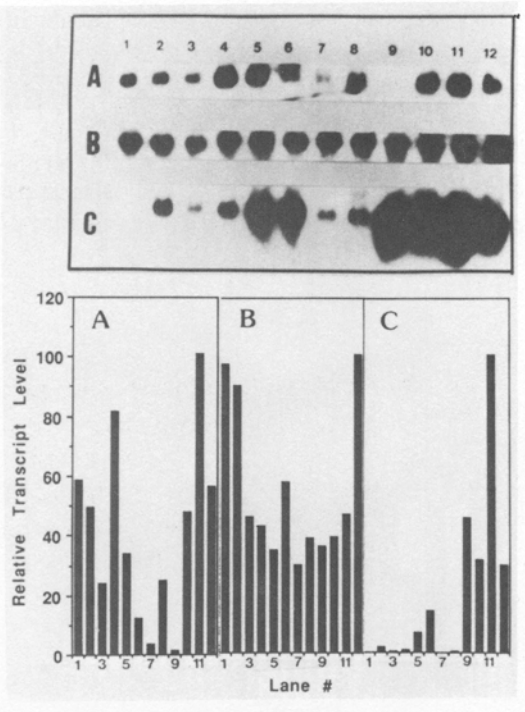


*Fig. 6.* Organ-specific expression of the *ubi3* gene in tomato. Each lane contains 8 μg of total RNA prepared from different organs of field-grown tomato plants. A, B, and C as in Fig. 5. Ripe fruit (stage 4), lane 1; mature green fruit (stage 2), lane 2; ripening fruit (stage 3), lane 3; immature green fruit (stage 1), lane 4; stems, lane 5; petioles from 4th leaves, lane 6; yellow flower petals, lane 7; yellow flower petals and ovaries, lane 8; 4th mature leaves, lane 9; pedicels, lane 10; young 1st leaves, lane 11; and green sepals, lane 12. Relative transcript level is defined in Fig. 5. The relative transcript level was arbitrarily set to 100 for the highest expressing sample.

*Immunoblot analysis of chloroplast proteins*

Since tomato *ubi3* lacked a transit peptide and was nearly identical to other proteins shown to be cytoplasmically located, there was no reason to believe that UBI3 was imported into chloroplasts. We explored the possibility that UBI3 shares structural similarity to another chloroplast protein. Conceivably, if a chloroplast protein were mutually recognized by both the PSI and UBI3 antibody, the PSI antibody might recognize UBI3 protein during the library screening. We examined whether there was any immunological cross reactivity between chloroplast proteins and antibodies against ubiquitin, the ubiquitin extension protein, and the PSI antibody from *Vicia faba*. The antibody specific for the UBI3 tail weakly cross-reacted with a single thylakoid protein of 65 kDa in tomato (Fig. 1, lane 1). The PSI antibody barely detected a protein of similar size (Fig. 1, lane 3). The weakness of the signal suggests that the antibodies against PSI and UBI3 recognize few if any common epitopes on chloroplast proteins. PSI antibody also did not detect up to 20 μg purified ubiquitin (data not shown).

Antibodies prepared against ubiquitin (supplied by Judy Callis) reacted with two bands in a bovine ubiquitin (Sigma) sample; the lower band corresponds to free ubiquitin (Fig. 7, lane 3). The pre-immune serum (also supplied by Judy Callis) did not react with the bovine protein. Free ubiq-

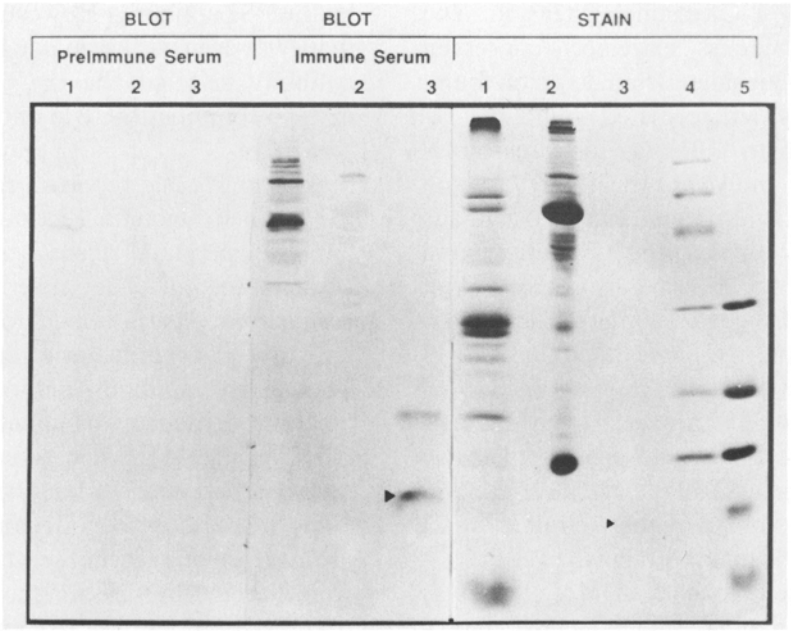


*Fig. 7.* Immunoblot analysis of spinach chloroplast proteins using ubiquitin antisera. Chloroplasts were treated with thermolysin as described [27]. Chloroplasts were re-isolated by spinning through 40% Percoll and washed in 0.33 M sorbitol, 50 mM Hepes-KOH pH 8.0. Samples were then fractionated into thylakoids (lane 1) and stroma (lane 2) after hypotonic lysis. Each lane represents fractions prepared from chloroplasts containing 20 μg chlorophyll and run on 15% SDS-PAGE. Lane 3 contains 1 μg bovine ubiquitin (Sigma). Lane 4: BioRad MW markers (97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa, 14.4 kDa). Lane 5: Enprotech MW markers (29 kDa, 20.4 kDa, 14 kDa, 6.1 kDa, 3.5 kDa). One set of samples were stained with Coomassie blue while parallel samples were transferred to nitrocellulose and probed with pre-immune serum or ubiquitin antiserum. The band corresponding to free ubiquitin is marked with a solid triangle.

uitin was not detected by the immune serum in protease-treated chloroplast samples from spinach. In a parallel experiment, the protease treatment was effective in completely degrading wheat germ translation products bound to the surface of chloroplasts (data not shown). Based on the sensitivity of the antibody to purified ubiquitin, we estimate that we would have detected ubiquitin in samples containing 1 ng/μg chlorophyll. The immune serum, however, reacted with higher molecular weight polypeptides found in both the thylakoid membranes and stroma (Fig. 7, lanes 1 and 2, respectively); most of the cross-reacting proteins were found in the thylakoids. The immunoreaction appears to be specific because it is unrelated to protein abundance (Fig. 7, compare immunoblot to stained gel). The immunoreaction was also observed with ubiquitin antibodies purchased from Sigma (data not shown). Furthermore, pre-immune serum showed little or no binding activity toward the polypeptides detected by the immune serum. The ability of membrane and soluble polypeptides to bind ubiquitin antibodies was also observed in protease-treated pea chloroplasts (data not shown).

## Discussion

Ubiquitin is encoded by at least four distinct genes in yeast all of which encode polyproteins [26]. In the present paper we report the sequence and expression characteristics of a plant homologue to the yeast gene *ubi3*. Plant homologues to all the yeast ubiquitin genes have recently been reported [2, 3, 4, 12, 13]. The tomato *ubi3* gene, like homologues in yeast, *Arabidopsis*, barley, *Drosophila*, and man, encodes a polyprotein consisting of

ubiquitin fused to a C-terminal extension. The ubiquitin polypeptide is extremely conserved varying only by 2–3 residues from ubiquitin found in other organisms. The predicted amino acid sequence of the tomato UBI3 tail most resembles that of barley and *Arabidopsis* (nearly 90% amino acid identity) but is also very related to humans (74% identity), *Drosophila* (69% identity), and yeast (65% identity). Given the high degree of conservation for this gene among the four organisms, it is reasonable to assume that tomato UBI3 is a ribosomal protein as was shown for yeast [9] and *Arabidopsis* [4]. In this regard it is interesting to note that the tomato *ubi3* promoter has five putative UAS elements and the overall consensus sequence is very similar to that found in yeast genes encoding ribosomal proteins.

Although the tomato *ubi3* cDNA clone was isolated using an antibody made against chloroplast proteins, all evidence suggests that the clone does not encode a chloroplast protein. Although the cDNA does not have a stop codon upstream of the first methionine, analysis of the corresponding genomic clone revealed that an in-frame stop codon occurs downstream of a potential initiating methionine. Primer extension studies also indicate that transcription initiation occurs downstream of any other potential translation initiation sites. These data indicate that the isolated clone does not encode a form of UBI3 having a transit peptide. The predicted protein is nearly identical to an *Arabidopsis* homologue shown to be localized in cytoplasmic ribosomes [4] and hence it is unlikely that UBI3 is targeted to the chloroplast.

Was the *ubi3* clone picked inadvertently? This possibility is diminished by the fact that the clone was only detected at a frequency of less than $10^{-5}$. We attempted to address this question by examining whether chloroplasts contain proteins structurally related to ubiquitin or the UBI3 tail. We found only one tomato chloroplast protein that was recognized by UBI3-specific antibody. The detected band is much larger than UBI3 and the cross-reactivity weak, indicating that the detected protein is structurally very different than UBI3. A similarly sized protein was also detected by the PSI antibody. However, the immunoreactivity was even weaker making it unlikely that PSI antibody selected the *ubi3* clone through an epitope common to UBI3 and a chloroplast protein.

In a previously reported case, St. John *et al.* [33] cloned ubiquitin genes using a monoclonal antibody prepared against the lymphocyte cell surface receptor. They determined that the cell surface receptor is bound to ubiquitin and the antibody was specific for an epitope on ubiquitin. Though the antibody did not recognize other ubiquitin conjugates or undenatured ubiquitin it did recognize SDS-denatured ubiquitin. In the case reported here, PSI antibodies did not detect 20 $\mu$g of purified SDS-denatured ubiquitin. We are aware of one other case where ubiquitin clones were selected by screening a library with an antibody that does not recognize purified SDS-denatured ubiquitin (Kirk Apt, personal communication). In this example, polyubiquitin genes from the red alga, *Callithamnion neglectum* were isolated by probing with an antibody made against purified phycobilisome proteins. One possibility we did not explore is whether the *E. coli*-expressed ubiquitin has a strong affinity for the antibody or one of the screening reagents. This affinity would need to be a property of the native or recombinant protein because SDS-denatured purified ubiquitin was not detected by the PSI or phycobilisome antibodies on western blots. PSI antibodies also did not detect SDS-denatured fusion protein.

Ubiquitin has not been found in mitochondria or bacteria [15]. Wettern *et al.* [36] recently reported evidence that ubiquitin or ubiquitin conjugates are found in *Chlamydomonas* chloroplasts. Furthermore, they speculated that ubiquitin might enter the chloroplast in the form of protein conjugates. At the time of this writing, they had not extended their observations to higher-plant chloroplasts (M. Wettern, personal communication). We were unable to detect any free ubiquitin with ubiquitin antibodies in chloroplasts from spinach or pea. However, in both organisms we did find higher-molecular-weight forms that reacted with the ubiquitin antibody, consistent with the observations of Wettern *et al.* [36]. The fact that pro-

tease treatment of intact chloroplasts does not diminish the signal indicates that the affected proteins are within the chloroplast. In lieu of the apparent absence of any chloroplast routing signals on ubiquitin proteins, the simplest explanation is that nuclear-encoded chloroplast proteins are ubiquitinated in the cytosol and are still capable of being imported into the chloroplast.

Further biochemical studies are required to establish whether the proteins detected by the antibody are indeed ubiquitinated and are not fortuitously cross-reacting with the antibody. If the proteins are ubiquitinated, it will be interesting to identify whether they are all nuclear-encoded. If chloroplast-encoded proteins are also ubiquitinated, it would be consistent with the recent report that lysed chloroplasts are capable of ubiquitinating chloroplast polypeptides [35]. The authors of this report speculated that a ubiquitin conjugating system may be involved in regulating protein turnover in chloroplasts. Conceivably, the ubiquitin for this process could be salvaged from imported conjugates. It will also be interesting to observe whether ubiquitination has an effect on the import of proteins into the chloroplast. In this context it is interesting to note the report that ubiquitination promotes the translocation of monoamine oxidase B into the mitochondrial outer membrane [37].

Other investigators have observed four size classes of ubiquitin transcripts expressed in most tissues of *Arabidopsis* and barley [2, 4, 11]. Probes specific for the extension protein genes only hybridize to the smallest class between 0.75–1.00 kb in *Arabidopsis* [4]. In tomato we observed only two size classes, of 1.6 and 0.8 kb transcripts, hybridizing to a general ubiquitin probe. We also found that a *ubi3* specific probe only hybridized to the 0.8 kb transcript. The larger transcript might be encoded by a homologue to the polyubiquitin gene, *ubq4* [2]. *Ubq4* encodes a 1.35 kb transcript in *Arabidopsis* [2]. Both size classes of tomato ubiquitin transcript were down-regulated by a 42 °C heat shock. Similarly, Burke *et al.* [2] previously found that 0.7 and 1.35 kb ubiquitin transcripts were down-regulated by heat shock in *Arabidopsis*. We did not observe, as they had found, that a 1.7 kb ubiquitin transcript was up-regulated by heat shock. In yeast, the *ubi4* gene is up-regulated and *ubi3* continues to be expressed during heat shock while *ubi1* and *ubi2* are down-regulated [10].

The tomato *ubi3* transcript is most abundant in light-grown and young tissues consistent with its presumed role in protein synthesis. We found that it was nearly absent in flower petals, mature light-grown leaves, and young light-grown leaves kept in darkness for three days. In contrast, the 1600 kb ubiquitin transcript showed high levels of expression in all samples. The different patterns of expression between the *ubi3* transcript and the 1600 kb ubiquitin transcript is further evidence for different functions for the proteins these transcripts encode, a ribosomal protein in one case and presumably a component of the proteolytic system in the other. Callis *et al.* [4] similarly found that levels of expression for the extension protein genes were lower in mature leaf tissue. Although they did not examine transcript levels in dark-adapted plants, they immunologically detected extension protein in dark-grown 3-day-old seedlings. Conceivably the expression of the extension protein in the dark-grown seedlings is developmentally programmed to coincide with a requirement for active protein synthesis during germination and seedling emergence whereas in slightly older photosynthetic tissue dark adaptation is a cue to curtail protein synthesis and hence to reduce expression of the extension protein.

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