

Isolation of a cDNA coding for an ubiquitin-conjugating enzyme UBC1 of tomato — the first stress-induced UBC of higher plants

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Abstract A clone of an ubiquitin-conjugating enzyme (UBC) was isolated from a λ -ZAP-cDNA library, generated from mRNA of tomato (*Lycopersicon esculentum*) cells grown in suspension for 3 days. The open reading frame called *LeUBC1*, encodes for a polypeptide with a predicted molecular mass of 21.37 kDa, which was confirmed by bacterial overexpression and SDS-PAGE. Database searches with *LeUBC1* showed highest sequence similarities to UBC1 of bovine and yeast. By Southern blot analysis *LeUBC1* was identified as a member of a small E2 subfamily of tomato, presumably consisting of at least two members. As revealed by Northern blot analysis *LeUBC1* is constitutively expressed in an exponentially growing tomato cell culture. In response to heat shock an increase in *LeUBC1*-mRNA was detectable. A strong accumulation of the *LeUBC1*-transcript was observed by exposure to heavy metal stress which was performed by treatment with cadmium chloride (CdCl_2). The cellular uptake of cadmium was controlled via ICP-MS measurements. The data suggest that like in yeast, in plants a certain subfamily of UBC is specifically involved in the proteolytic degradation of abnormal proteins as result of stress.

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Key words: Ubiquitin-conjugating enzyme (E2); UBC gene family; CdCl_2 -treatment; heat shock; *Lycopersicon esculentum*

1. Introduction

The covalent attachment of the protein ubiquitin to other cellular proteins has been implicated in a number of important physiological processes including selective protein degradation, DNA repair, cell cycle control, and organelle biosynthesis [1]. This pathway is highly conserved in all eukaryotes. Ubiquitin becomes attached to substrate proteins in three enzymatic steps: (i) Ubiquitin is activated by an ATP-dependent thiol coupling of its carboxyterminus to an activating enzyme (E1); (ii) a subsequent transthioleation leads to the transfer of ubiquitin from E1 onto a ubiquitin-conjugating enzyme (E2). (iii) Finally, ubiquitin is transferred to an ϵ -amino group of an internal lysine residue of an appropriate target protein. In some cases, this transfer requires a third type of enzymes, called ubiquitin-protein ligases (E3).

E2s constitute a heterogeneous family of enzymes differing in some biochemical properties as (i) molecular mass, (ii) in vitro substrate specificities, and (iii) requirement for E3 [2]. Most data on E2 exist for yeast. Here genetic analysis revealed more than eleven different E2 gene products. They could be divided into several subfamilies, which exhibit specific physiological functions in vivo. There is one E2-subfamily, containing *ScUBC1*, *ScUBC4*, *ScUBC5*, *ScUBC7*, which mediate essential functions in cell growth and viability under stress. So, transcripts of *ScUBC4* and *ScUBC5* are induced by heat stress and of *ScUBC7* by exposure to cadmium. Additionally, *ubc4 ubc5* double mutants are inviable under heat shock conditions, and the *ubc7* single mutant is cadmium hypersensitive. This suggests that both stresses generate abnormal proteins which are normally subject to *ScUBC4*, *ScUBC5* and *ScUBC7*-mediated proteolysis [1,3–5].

Beneath differences in physiological functions E2s could be divided into two structural classes. All E2s known so far contain a conserved domain of about 16 kDa, called UBC domain. This domain includes a central cysteine residue which is required for the formation of the thiol ester [1]. E2s containing only this domain belong to class I enzymes. In the case of class II or class III E2 enzymes additional N-terminal or C-terminal extensions exist, respectively, to this core [1]. These elements are thought to be responsible for enzyme specificity or regulation.

At least in plants the E2 family constitutes of several homologues members [6,7]. In *Arabidopsis thaliana* 14 genes (designated *AtUBC1*–14) have been isolated. They could be divided into six subfamilies based on sequence comparison [8–11]. For plant UBCs different expression in developmentally regulated events such as flower development, cell cycle progression, leaf senescence or peroxisome assembly has been found [12–15]. But so far no plant UBCs have been described, which are significantly regulated upon stress [6,7].

Here we describe a cDNA encoding for an E2 of tomato (*LeUBC1*) and show first for plants that in tomato cells grown in suspension, its transcript is upregulated by cadmium stress and heat shock. By sequence comparison it is shown that the cDNA clone coding for tomato UBC1 reveal highest homology to yeast and bovine UBC1.

2. Materials and methods

2.1. Plant material

Tomato (*Lycopersicon esculentum* cv. Lukullus) cells were grown in suspension as described before [16]. $2 \cdot 10^7$ mid-log phase cells per 15 ml were inoculated into 100 ml fresh nutrient medium containing 30 g/l sucrose, cultivated on a rotary shaker (150 r.p.m.) at 29°C without light.

For heavy metal stress CdCl_2 was added to a final concentration of 100 μM at the second day of culturing. At times indicated cells

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This paper is dedicated to Prof. Dr. B. Parthier on the occasion of his 65th birthday.

The nucleotide sequence reported in this paper has been submitted to the GenBank[®]/EMBL Data Bank with accession number X82938.

A

Linker
[GGCTGCAGG]AA

TTCGGCACGAGGGCGACTTTTGCATAAACCAAAATTAGAATCAAATTGGAAGAGAGAAAAAAA

1 ATG GTG GAC TTG GCT AGG GTT CAA AAG GAG CTC CAT GAA TGC AAC AGA
1 M V D L A R V Q K E L H E C N R

49 GAT GTT CAG GTT TCT GGA ATT AAT GTT ACC CTT AAA GGT GAC AGT CTC
17 D V Q V S G I N V T L K G D S L

97 ACT CAC TTG ATT GGT ACA ATC CCT GGT CCT GTT GGT ACT CCT TAC GAA
33 T H L I G T I P G P V G T P Y E

145 GGC GGT ACT TTC AAG ATC GAT ATC ACT CTT ACT GAT GGC TAC CCA TTT
49 G G T F K I D I T L T D G Y P F

193 GAG CCT CCA AAA ATG AAA TTC GCC ACA AAA GTT TGG CAT CCC AAC ATA
65 E P P K M K F A T K V W H P N I

241 AGT AGT CAA AGT GGA GCA ATA TGC CTA GAC ATC CTG AAG GAC CAG TGG
81 S S Q S G A I C L D I L K D Q W

289 AGC CCA GCA CTA ACT CTC AAG ACA GCT CTC CTT TCT ATA CAA GCA TTA
97 S P A L T L K T A L L S I Q A L

337 CTT TCT GCT CCT GAA CCT GAT GAT CCA CAA GAT GCA GTT GTT GCA CAG
113 L S A P E P D D P Q D A V V A Q

385 CAG TAT CTT AGA GAA CAT CAG ACC TTT GTC GGC ACA GCT CGT TAC TGG
129 Q Y L R E H Q T F V G T A R Y W

433 ACT GAG ACT TTT GCA AAA ACA TCC ACA CTT GCT GCA GAC GAC AAG ATA
145 T E T F A K T S T L A A D D K I

481 CAA AAG CTT GTG GAA ATG GGC TTT CCT GAA GCT CAA GTG AGG AGT ACT
161 Q K L V E M G F P E A Q V R S T

529 TTG GAA GCA AAT GGT TGG GAT GAA AAC ATG GCT CTT GAA AAG CTG TTG
177 L E A N G W D E N M A L E K L L

577 TCC AGC TAA
193 S S Ter

586 AACCCCTTCTACTGCAACTCATATTTTGATAAGACAATTATATCCTTCCAGCAAAAGCTGATGA
649 CTAGAATAGAGTCACTCGGTTATACTGTTGCTTGGCAATCTTGTTTCTGTCTCCTTTATGGTT
712 TGCTGTTGACATCTCTTCATATCTGTGAAGATTCTGATGTTATTTTACAATATCAAGCAAA
775 TTGCATATGAATCATGGGGAGGAAGTGGACTTCCGGGGTGAAAAAAAAAAAAAAAAAAAA
838 AAAAAAAAAAAAAAAAAAAAAAAAAAA[CTCGAGGGGG]
Linker

B

	Amino acid comparison	
	of the conserved position around the active site cysteine residue	of the full sequence (similarity in aa overlap)
	↓	
LeUBC1 (tomato)	H P N I S S Q S G A I C L D I L K D Q W	
UBC1 (bovine) [25]	H P N I S S V T G A I C L D I L K D Q W	84.3 % (197 aa)
ScUBC1 (yeast) [3]	H P N I S S V T G A I C L D I L K N A W	80.1 % (191 aa)
UBC (tomato) [14]	H P N I N S - N G S I C L D I L K E Q W	81.5 % (146 aa)
ScUBC4 (yeast) [4]	H P N I N A - N G N I C L D I L K D Q W	84.0 % (144 aa)
AtUBC11 (A.thal.) [9]	H P N I N S - N G S I C L D I L K E Q W	83.4 % (127 aa)
ScUBC5 (yeast) [4]	H P N I N S - S G N I C L D I L K D Q W	86.0 % (121 aa)
EST, Z25704 (A.thal.)	- P N I S S Q S G A I C L D I L K D Q W	96.5 % (114 aa)

Fig. 1. Primary structure of clone A7. A: nucleotide sequence and derived amino acid sequence of clone A7. The open reading frame encodes for a protein of M_r 21.37. Nucleotides and amino acid residues are numbered on the left. By sequence comparison the protein was identified as E2 homologue of tomato, called *LeUBC1* (E2_{21K}). The amino acids of the highly conserved region of the active site region of E2s are underlined. The active site cysteine residue for binding of the ubiquitin and a possible phosphorylation site of protein kinase C ('T A R') are in bold. B: amino acid comparison between tomato E2_{21K} and other described E2s. The arrow indicates the active site cysteine residue.

←

were harvested, washed twice with 50 ml culture medium containing 100 μ M EDTA and were subsequently used for RNA extraction and CdCl₂ estimation.

For heat shock 3-day-old tomato cell cultures were treated subsequently as follows: variant I: 1 h 37°C; 2 h 38,5°C; 1 h 40°C; variant II: 15 min 40°C; 2,5 h 25°C; 2 h 40°C.

2.2. Extraction of DNA and RNA

High-molecular weight tomato DNA was isolated according to the protocol of Wienand and Feix [17]. Plasmid DNA was isolated using the QUIAGEN Plasmid Kit (QUIAGEN) for maxipreparation, and the Magic[®] Miniprep DNA Purification System (Promega) for minipreparations, according to the supplier's instructions. Total RNA was extracted by phenol/chloroform/iso-amylalcohol treatment as described before [18] using modifications of Andresen et al. [19]. Poly(A⁺)-RNA was isolated by oligo-dT chromatography according to [20].

2.3. Northern and Southern analysis

RNA electrophoresis (40 μ g total RNA per lane), and Northern analysis with *LeUBC1*-specific cDNA probes were performed according to [21]. After the transfer of RNA onto nitrocellulose BA 85 filters (Schleicher and Schüll, Dassel, Germany), filters were hybridized with a Digoxigenin-dUTP-labeled cDNA insert prepared from the plasmid p*LeUBC1*. Labeling was achieved by Random primed DNA synthesis using the DIG-DNA-labeling kit (Boehringer, Mannheim, Germany). Hybridization and detection were carried out according to Engler-Blum et al., 1993 [22], by assaying alkaline phosphatase via the chemiluminescence substrate CSPD (Bedford, USA). Estimation of RNA sizes was based on the RNA ladder 0.24–9.5 kb of GIBCO BRL. For Southern blot analysis, 8 μ g of genomic DNA was digested with the restriction enzymes *Eco* RI, *Eco* RV or *Hind* III. Agarose gel electrophoresis and Southern blotting were performed as described by [21]. Hybridization and detection were carried out according to [22].

2.4. Construction and screening of cDNA library, cDNA sequencing

Poly(A⁺) containing RNA (5 μ g) from 3-day-old tomato cell culture was transcribed into double-stranded cDNA using the ZAP-cDNA Synthesis Kit (Stratagene, Heidelberg, Germany) according to the supplier's instructions. The resulting cDNA was ligated into the Uni-ZAP XR vector, packaged in vitro using Gigapack II plus packaging extract (Stratagene) according to the supplier's protocol. The Uni-ZAP XR library was first amplified and then screened with an antibody raised against purified Ap₄A hydrolase of tomato [23]. DNA of positive clones was isolated and cloned into the pBlue-script SK (Stratagene) by standard methods [21]. Internal restriction sites were used to generate subclones for sequencing. Plasmid DNA was purified with StrataClean Resin (Stratagene) and then sequenced according to the method of Sanger with the T⁷sequencing Kit (Pharmacia).

2.5. Overexpression in *Escherichia coli*

The vector pBlue-script SK and the host strain *Escherichia coli* XL1-Blue were used to overexpress the cDNA insert of the clone p*LeUBC1*. *E. coli* XL1-Blue was transformed with pBlue-script SK, both with and without the cDNA insert. Total proteins of isopropyl- β -thiogalactopyranoside (IPTG)-induced or non-induced cultures were isolated by sonification in cracking buffer (60 mM Tris-HCl, pH 6.8, 1% (w/v) 2-mercaptoethanol 1% (w/v) SDS, 10% (w/v) glycerol, 0.1% (w/v) bromophenol blue) according to [24]. After heating the samples at 95°C for 5 min, the insoluble cell debris was removed by centrifugation at 10,000 g for 5 min. Protein solution was separated by SDS-PAGE and stained by Coomassie.

2.6. Estimation of the cadmium content of cells

Cells of suspension cultures grown for times indicated were dried at 60°C during 24 h, and dry weights were determined. Samples of 20–100 mg dried material were acid-digested separately with 6 ml con-

centrated nitric acid (65% suprapur, Merck, Darmstadt, Germany) in a microwave oven (MDS 205, CEM Corporation, Matthews, USA) and diluted to a final volume of 25 ml with distilled water. Cadmium content of cells was analyzed by inductively coupled plasma mass spectrometry using a PQ2+ (VG Elemental, Winsford, UK). For quantitative analysis calibration samples were prepared from a commercial standard solution of cadmium (Merck, Darmstadt, Germany). An Indium solution (Johnson Mattheys, Karlsruhe, Germany) was used as internal standard.

3. Results and Discussion

3.1. Isolation, sequencing and identification of *LeUBC1* — a cDNA encoding *LeE2_{21K}*

LeUBC1 was isolated from a ZAP-cDNA library which was constructed from poly(A⁺) mRNA of tomato (*Lycopersicon esculentum*) cells grown in suspension for 3 days. By immunoscreening in an unrelated study one positive clone, called A7, was isolated out of 1·10⁶ recombinant λ -phages.

The full-length cDNA clone A7 contained a 5' flanking region of 65 bp and a 3' untranslated region of 279 bp, in-

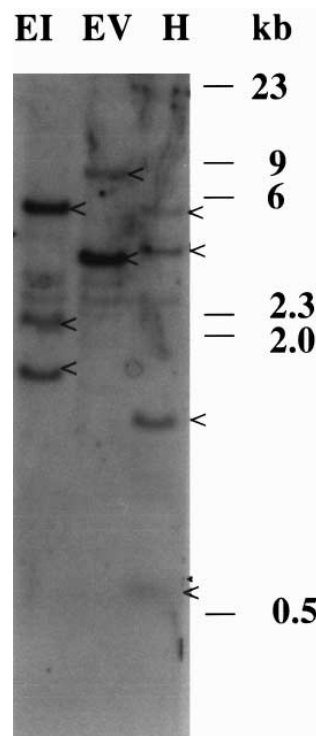


Fig. 2. Southern blot analysis of tomato genomic DNA with the *LeUBC1* cDNA probe. 8 μ g genomic DNA was digested with the restriction endonucleases *Eco* RI (E I), *Eco* RV (E V) and *Hind* III (H), fractionated by agarose gel electrophoresis, and transferred onto nylon membranes. The blot was hybridized with DIG-labeled *LeUBC1* cDNA for 12 h at 65°C. Following a wash with 20 mM Na₂HPO₄, 1 mM EDTA, 1% SDS (3×20 min, 65°C), the duplexes were detected by Anti-DIG-antisera coupled with alkaline phosphatase and the chemiluminescence substrate CSPD (Bedford, USA).

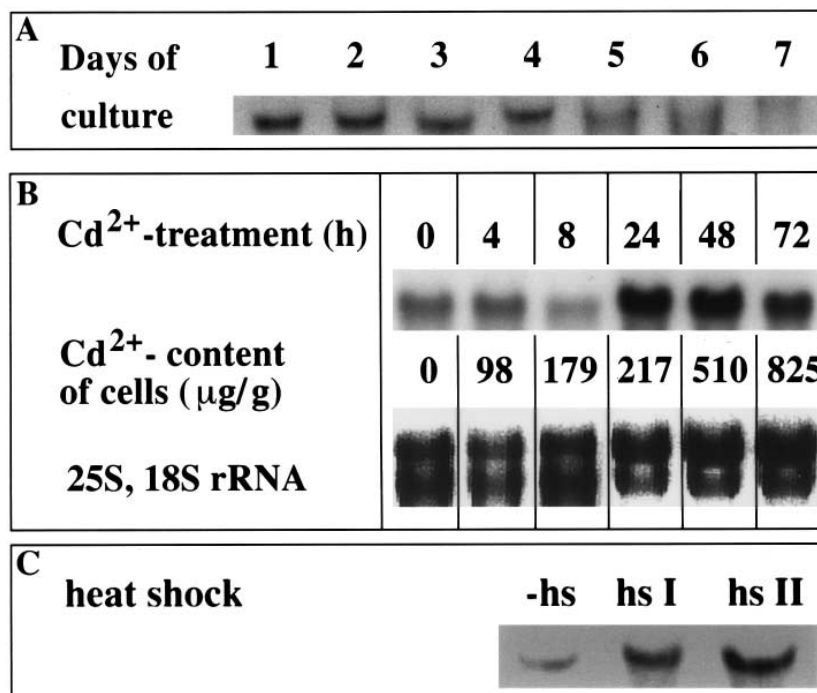


Fig. 3. Northern blot analysis of *LeUBC1* mRNA accumulation during growth of the tomato cell suspension culture (A), during heavy metal stress by cadmium (B), and in response to heat shock (C). For A, total RNA of tomato cells grown in suspension for times indicated were extracted and processed as mentioned below. For B, 24 h old cell cultures were supplied with CdCl_2 to a final concentration of 100 μM , harvested after distinct times of incubation (4, 8, 24, 72 and 96 h), washed twice with medium containing 100 μM EDTA and were subsequently used for estimation of cadmium content by ICP-MS or for RNA preparation. As control in the lower trace the range of 25S, 18S rRNA of the ethidium bromide stained gel is shown before blotting. C: heat shock was given subsequently to 3-days-old tomato cell cultures using the following two types of treatment; hs I: 1 h 37°C; 2 h 38.5°C; 1 h 40°C; hs II: 15 min 40°C; 2.5 h 25°C; 2 h 40°C. For Northern blot analysis 40 μg total RNA/lane was separated electrophoretically on 1.5% agarose gels, transferred onto nylon membrane and hybridized with the DIG-labeled *LeUBC1* cDNA (12 h, 65°C). Following a wash with 20 mM Na_2HPO_4 , 1 mM EDTA, 1% SDS (3×20 min, 65°C), the heteroduplexes were detected by Anti-DIG-antisera coupled with alkaline phosphatase and the chemiluminescence substrate CSPD (Bedford, USA). The approximate size of the transcript is 0.75 kb.

cluding 49 bp of the poly(A) tail. There was no obvious polyadenylation signal sequence. The open reading frame of clone A7 encoded for 194 amino acids which corresponds to a molecular mass of 21.37 kDa (Fig. 1A).

Comparison of the nucleotide and the deduced amino acid sequence of A7 with sequences of data bases (EMBL, Heidelberg, Germany) using TFASTA algorithm revealed strong homology to UBCs (E2). As shown in Fig. 1B, on amino acid level the clone A7 exhibited 47.2% identity and 84.3% similarity, respectively, over 197 amino acids to UBC1 (E2_{25K}) from bovine (S51016) [25], and 44.5% identity and 80.1% similarity, respectively, over 191 amino acids to ScUBC1 from yeast (X56402) [3]. Because of this degree of homology the clone A7 was designated as *LeUBC1* (*Lycopersicon esculentum* UBC1), and the corresponding protein was designated as LeE2_{21K}. Two other E2s of tomato, *LeUBC* (X73419) [14] and *Le1KUC* (L23762) has been described previously. The homologies between *LeUBC1* and these tomato UBCs cover only the UBC domain of 146 amino acids, carrying 45.9% identity and 81.5% similarity, respectively, in case of *LeUBC* and 44.3% identity and 81.2% similarity, respectively, in case of *Le1KUC*. Other plant UBCs show up sequence homologies over smaller sequence parts. The one with highest homologies, *AtUBC11* of *Arabidopsis thaliana* shows 48.0% identity and 83.4% similarity in 127 amino acids overlap. So *LeUBC1* seems to be a plant counter part of bovine E2_{25K} (UBC1). All other plant UBCs are not closely related in amino acid

sequence to bovine E2_{25K} [11], an E2 which is able to synthesize multiubiquitin chains in vitro [25]. Interestingly, an EST of *Arabidopsis thaliana* has been described (Z25704) which codes for the uncomplete 3' end of an additional E2 member. It shows 76.3% identity and 96.5% similarity in 114 amino acids overlap. This uncomplete sequence might be at least the only plant homologue to *LeUBC1* known so far.

Based on the structural classification of E2s described by Jentsch [1], tomato E2_{21K} identified here, can be regarded as a class II UBC like the bovine UBC1 and yeast ScUBC1. Generally, E2s carry a cysteine residue in a highly conserved region of about 20 amino acids [1]. It is assumed that ubiquitinylation takes place at this cysteine residue via a thiol ester. The sequence of tomato E2_{21K} carries cysteine residues in position 14 and 88. But only cysteine-88 is placed in a region strongly conserved in other known E2s. This putative active site of tomato E2_{21K} shows 90% identity (18 of 20 amino acids) towards the region of bovine E2_{25K} [25] and similar high homology to yeast E2s like ScUBC1 [3], ScUBC5 (X17494) [4], or ScUBC4 (X17493) [4] and to E2 from tomato like *LeUBC* [14] or from *Arabidopsis thaliana* like *AtUBC11* (Z14992) [9] (Fig. 1B).

Upon overexpression of the *LeUBC1* in *E. coli*, separation of the recombinant protein by SDS-PAGE, a molecular mass of 22 kDa was determined which closely corresponds to that predicted from the deduced amino acid sequence (data not shown).

3.2. *LeUBC1* — a member of a small UBC-gene family

In Southern blot analysis of tomato DNA digested with the restriction endonucleases Eco RI, Eco RV and Hind III up to four fragments were detected by hybridization with the *LeUBC1* cDNA (Fig. 2). This indicates, that *LeUBC1* is one member of a small E2 subfamily, potentially consisting of at least two members.

3.3. *LeUBC1* mRNA accumulates in tomato cells stressed by heat shock or heavy metals

UBCs are known to be expressed in response to various environmental factors [1]. Interestingly, this expression is specifically regulated at least for each isoenzyme. A transient occurrence upon stresses or during different developmental stages accords with their suggested role to attribute for a specific breakdown of proteins. Several plant E2s were described, but functional implications are still lacking. Therefore, we examined expression of tomato UBC1 during growth of cells cultured in suspension. Subsequently, we analyzed the effect of heavy metal stress and of heat stress on the expression of *LeUBC1*.

As revealed by Northern blot analysis (Fig. 3A), *LeUBC1* is expressed constitutively during logarithmic growth of tomato cells (days 1–4), whereas *LeUBC1* mRNA amount clearly declined in the stationary phase of culturing (days 4–7). At day 7 *LeUBC1* mRNA was hardly detectable. The pattern of expression is similar to that of yeast *ScUBC4*, but in contrast to the expression of *ScUBC1* which is induced in the stationary phase [3,4].

In order to test a possible role of *LeUBC1* in stress responses of tomato cells we analyzed heavy metal stress and heat stress. First, logarithmically growing cells at day 1 of culturing were treated with 100 μ M CdCl₂. As indicated by the content, cadmium was accumulated within the growing cells steadily during treatment (Fig. 3B). After 72 h of treatment 825 μ g cadmium/g dry weight were detected by ICP-MS. Northern blot analysis revealed strong accumulation of *LeUBC1* mRNA upon heavy metal stress (Fig. 3B). Interestingly, accumulation of the *LeUBC1* mRNA occurs first at 24 h of cadmium treatment. This indicates that only a certain threshold in the amount of cadmium taken up is able to induce *LeUBC1* expression. In the yeast UBC system transcription of *ScUBC5* and *ScUBC7* is strong induced by cadmium, whereas *ScUBC1* and *ScUBC4* are only moderately induced [5]. Treatment (100 μ M) for about one generation time (20–30 min) leads to strong induction of *ScUBC7*.

We also examined expression of *LeUBC1* under heat shock conditions. Three-day-old tomato cell cultures were treated by two different variants of heat stress (variant I: 1 h 37°C; 2 h 38,5°C; 1 h 40°C; variant II: 15 min 40°C; 2,5 h 25°C; 2 h 40°C). Both variants of heat shock induced transcription of *LeUBC1*, but variant II exhibited a somewhat stronger *LeUBC1* expression.

Taken together *LeUBC1* exhibit at least for plants two

unique properties: (i) stressed tomato cells respond by a strong accumulation of *LeUBC1*-transcript. (ii) *LeUBC1* is closely related to bovine and yeast UBC1 in terms of sequence similarity. Thus, *LeUBC1* is supposed to be a member of a new plant E2-family. Our data support the assumption that UBCs function in an ubiquitously occurring system of protein degradation, activated during environmental stresses.

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