The Arabidopsis thaliana UBC7/13/14 Genes Encode a Family of Multiubiquitin Chain-forming E2 Enzymes*

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Covalent modification of proteins by attachment of multiubiquitin chains serves as an essential signal for selective protein degradation in eukaryotes. The specificity of ubiquitin-protein conjugation is controlled in part by a diverse group of ubiquitin-conjugating enzymes (E2s or UBCs). We have previously reported that the product of the wheat TaUBC7 gene recognizes ubiquitin as a substrate for ubiquitination in vitro, catalyzing the condensation of free ubiquitin into multiubiquitin chains linked via lysine 48 (van Noeker, S., and Vierstra, R. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10297-10301). Based on this activity, this E2 may play a central role in the ubiquitin proteolytic pathway by assembling chains in vivo. Here, we describe the cloning and characterization of a three-member gene family from Arabidopsis thaliana (designated AtUBC7/13/14) encoding structural homologs of TaUBC7. Like TaUBC7, recombinant AtUBC7/13/14 proteins formed multiubiquitin chains in vitro. AtUBC7/13/14 mRNAs were found in all tissues examined, and unlike related UBCs from yeast, the levels of mRNA were not elevated by heat stress or cadmium exposure. Transgenic Arabidopsis were engineered to express increased levels of active AtUBC7, for the first time altering the level of an E2 in a higher eukaryote. Plants expressing high levels of AtUBC7 exhibited no phenotypic abnormalities and were not noticeably enriched in multiubiquitin conjugates. These findings indicate that the in vivo synthesis of multiubiquitin chains is not rate-limited by the abundance of AtUBC7 and/or involves other, yet undefined components.

A variety of essential processes in eukaryotes are regulated by selective protein breakdown via the ubiquitin-dependent proteolytic pathway (Hershko and Ciechanover, 1992, Vierstra, 1993; Ciechanover, 1994). In this pathway, protein targets are first modified by attachment of a chain of ubiquitin monomers, internally linked through Lys48 of one ubiquitin and the carboxyl-terminal Gly76 of the adjacent ubiquitin. This modification serves as a signal for the subsequent breakdown of the target protein by the 26S proteasome complex (Hershko and Ciechanover, 1992). A 26S proteasome subunit which recognizes multiubiquitin chains has been described recently and appears to have a high affinity for chains containing four or more ubiquitins (Deveraux et al., 1994; van Noeker et al., 1996).

Substrates of the ubiquitin-dependent proteolytic pathway include aberrant polypeptides and important cellular regulators such as phytochrome A (Jabben et al., 1989), cyclins (Glotzer et al., 1991), p53 and c-jun oncoproteins (Scheffner et al., 1993; Treier et al., 1994); the yeast MATα2 transcriptional regulator and G protein Gpa1 (Hochstrasser et al., 1991; Madura and Varshavsky, 1994), and components of the NF-κB transcriptional complex (Palombella et al., 1994).

Ubiquitin conjugation is an ATP-dependent, multi-step process requiring the sequential action of at least two enzymes (Hershko and Ciechanover, 1992). Ubiquitin-conjugating enzymes (E2s)1 function by accepting activated ubiquitin from ubiquitin activating enzymes (E1s) and transferring it to a target protein. This transfer involves the formation of an ubiquitin-E2 thiol-ester intermediate where ubiquitin is linked to a specific cysteine within E2. The bound ubiquitin then becomes conjugated to a target protein via an isopeptide bond between Gly76 of ubiquitin and free lysyl ε-amino groups within the target. In at least some cases, additional recognition factors, termed ubiquitin-protein ligases (E3s), are required for substrate recognition and ubiquitin transfer (Ciechanover, 1994; Scheffner et al., 1995).

Proteins destined for degradation via the ubiquitin-dependent proteolytic pathway become multiubiquitinated by the attachment of a chain of ubiquitin monomers linked through Gly76 → Lys48 (Chau et al., 1989). The mechanism by which this intermolecular linkage is formed in vivo is unknown, but at least two possibilities exist. In a two- or multi-step process, a single ubiquitin is attached to a target protein; this ubiquitin would then serve as the site for appending additional ubiquitins. Alternatively, multiubiquitin chains could be formed by concatenation of free ubiquitin monomers; the resulting chain would then be attached to the target in a single step (Chen and Pickart, 1990; van Noeker and Vierstra, 1993). In support of the latter possibility are the observations that (i) several types of E2s exist that catalyze the formation of free multiubiquitin chains, at least in vitro; (ii) free chains can interact with E1 and various E2s and be transferred en masse to targets in vitro with kinetics similar to ubiquitin monomers; and (iii) large cellular pools of free multiubiquitin chains exist in a variety of eukaryotes (Chen and Pickart, 1990; van Noeker and Vierstra, 1991, 1993).

We have previously reported the cloning of a cDNA from

1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-initiated polymerase chain reaction; bp, base pair(s).
Here, we report the isolation and characterization of a three-
Ta
cloned the corresponding genes from the plant Arabidopsis
thaliana, which is better suited for genetic manipulations.
In an attempt to better elucidate the role of E2s such as
TaUBC7 in mult ubiquitin chain formation in vitro, we have
cloned the corresponding genes from free ubiquitin in vitro. Transgenic Arabidopsis were en-
gineered to express high levels of AtUBC7. This ectopic expres-
sion did not phenotypically alter the plants nor increase the
pool of mult ubiquitin chains. From this, we conclude that
mult ubiquitin chain formation in vitro is not limited by
AtUBC7/13/14 enzyme levels.

MATERIALS AND METHODS

Isolation of Clones for Members of the Arabidopsis AtUBC7 13/14
Gene Family—Unless otherwise indicated, A. thaliana L., ecotype Co-
lumbia was used as the source of all DNA and RNA. A 577-bp EcoR II/Scal fragment from the wheat cDNA, TaUBC7 (van Nocker and Vier-
stra, 1991), was used to probe an amplification-1-ZAP (Stratagene, La Jolla, CA) cDNA library prepared with poly(A)+ RNA isolated from green leaves (Callis et al., 1990). The probe was radio-labeled with 32P-labeled dCTP by the random priming method (Feinberg and Vogelstein, 1983) and hybridized to membrane-bound DNA as suggested by the manu-
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E. coli strain XL-1 Blue MRF’ (Stratagene). This screen resulted in the identification of the cDNA
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To isolate the AtUBC7-like cDNAs AtUBC13 and AtUBC14, we screened an amplified, λ-ZAP II cDNA library prepared with 0.5–1-
kilobase pair poly(A)+ RNA isolated from 3-day-old etiolated, hypocotyl/cotyledon tissue pretreated with ethylene (Schindler
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In all cases, DNA sequence was determined from both strands (Vieira
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RESULTS

Isolation and Characterization of the AtUBC7/13/14 Genes—As a first step toward elucidating the mechanism(s) used to synthesize multiubiquitin chains in Arabidopsis, we identified Arabidopsis homologs of wheat TaUBC7, an E2 capable of forming multiubiquitin chains in vitro. A fragment of the TaUBC7 cDNA was used as a probe to screen an amplified λ-ZAP cDNA expression library synthesized with mRNA isolated from Arabidopsis leaf tissue. Examination of 2.5 × 10^5 phage yielded a new 783-bp cDNA (designated AtUBC7; Fig. 1) related to TaUBC7 but not to any previously characterized AtUBC genes (AtUBC1–6, Sullivan et al. (1994); AtUBC8–12, Girod et al. (1993); and AtUBC15 (formerly AtUBC2-1), Bartling et al. (1993)). The AtUBC7 cDNA ended in a poly(A) tract of 40 residues, preceded within 30 bases by several potential polyadenylation consensus sequences (Dean et al., 1986). A single long open reading frame was identified that encoded a 18,722-Da protein of 166 amino acids with a predicted isoelectric point of 5.33 (Figs. 1 and 2). The derived amino acid sequence exhibited homology with the wheat TaUBC7 gene product (76% identity, 85% similarity (van Nocker and Vierstra, 1991)) and with the yeast E2, TaUBC7 amino acid sequence exhibited homology with the wheat ASAPROBETOSCREENAL sequences, a 484-bp translated sequences (data not shown).

Purification of E2s from Arabidopsis—Crude protein extracts (see above) were prepared from green leaves and clarified by centrifugation at 20,000 × g for 20 min. Protein precipitating between 40 and 70% ammonium sulfate was resuspended in 50 mM Tris-HCl (pH 8.0, 4 °C) containing 1 mM Na_2EDTA. The resulting extract was applied to DE52 cellulose, and E2s were eluted in a 100–150 mM step of KCl in 50 mM Tris-HCl (pH 8.0, 4 °C) containing 0.5 mM dithioerythritol. All protein purification steps were carried out at 0–4 °C.

The derived amino acid sequence of AtUBC7 is shown above the corresponding nucleotide sequence. The nucleotide sequences represent the longest cDNAs isolated. Gaps in the sequence alignments are indicated by underlined asterisk. Translation stop codons (TGA) are indicated by an arrowhead.
Examination of 1.5 \times 10^5 phage resulted in the detection of a genomic sequence encompassing the entire cDNA of AtUBC7. An additional clone, designated AtUBC13, was identified that contained AtUBC7-related sequence (above). To identify a genomic equivalent of the AtUBC14 cDNA, the Arabidopsis genomic library was screened using a 196-bp XbaI/HindIII fragment containing AtUBC14 non-translated sequence as a probe. This screen resulted in the detection of a genomic sequence that included the entire cDNA sequence of AtUBC14.

The predicted protein products of the AtUBC13/14 genes were nearly identical to AtUBC7 in terms of both apparent molecular mass and charge (AtUBC13: M_r = 18,822, pI = 5.34; AtUBC14: M_r = 18,728, pI = 5.33). All three contain a conserved cysteine (residue 89 for AtUBC7) at a position similar to that of the active-site cysteine in the yeast E2 RAD6.

**Fig. 2.** Amino acid sequence relationships between the members of the AtUBC7/13/14 protein family and other E2s. A, amino acid sequence alignment of the AtUBC7/13/14 proteins with wheat TaUBC7 and yeast ScUBC7. Residues which are identical or similar to AtUBC7 are outlined in black or gray, respectively. The 13-residue insertion characteristic of the AtUBC7-type E2s is underlined. The presumed active-site cysteine (Cys-89 for AtUBC7) is marked with an arrow. Numbers refer to the amino acid position for AtUBC7. B, dendrogram of amino acid sequence relationships among the Arabidopsis AtUBC7/13/14 protein family, bovine E2–25 kDa, and other E2s. Distance along the horizontal axis separating two sequences is proportional to the divergence between the sequences. Members of the yeast ScUBC4/5 family, the Arabidopsis AtUBC8–12 family, and the human UBC5a/b/c family are included as an example of closely related E2 protein families from different kingdoms. Sequences shown are Arabidopsis AtUBC7/13/14 (this work) and AtUBC8–12 (Girod et al., 1993), wheat TaUBC7 (van Nocker and Vierstra, 1991), yeast ScUBC7 (Jungmann et al., 1993), CDC34 (Goebl et al., 1988), and ScUBC4/5 (Seufert and Jentsch, 1990), human UBC5a/b/c (Jensen et al., 1995), tomato UBC1 (K. Feussner and C. Wasternack, unpublished data), and bovine E2–25 kDa (Chen et al., 1991).
wheat TaUBC4, and Arabidopsis AtUBC1 (Sung et al., 1990; Sullivan and Vierstra, 1991), implicating this cysteine in the formation of the thiol-ester bond with ubiquitin. The region surrounding this cysteine conformed to the E2 active site motif HPN(I/V/X)2GX(I/V/L)C(I/L)X(I/V)(I/L) that is present in almost all E2s characterized to date. Additional cysteines are present at positions 17 and 158 in all three E2s, and at position 6 in AtUBC13. AtUBC7/13/14 contains a conserved 13-residue sequence (GDDPXGYELASER) carboxy-terminal to the active site cysteine; a similar insertion is present also in wheat TaUBC7 and yeast ScUBC7 and ScUBC3 (CDC34). Pairwise comparisons of AtUBC7/13/14 revealed a percent deduced amino acid sequence identity of 86–96%. In contrast, AtUBC7/13/14 exhibited at most only 58% amino acid sequence identity to the other Arabidopsis E2s that are fully characterized to date (Bartling et al., 1993; Girod et al., 1993; Sullivan et al., 1994). Because of the structural similarity between AtUBC7, -13, and -14, we propose that they constitute a functionally related family of E2s in Arabidopsis.

The complete nucleotide sequences of the AtUBC7/13/14 genomic clones were determined and their structures are depicted in Fig. 3. Five introns exist within the region corresponding to the cDNAs of AtUBC7 and -13, whereas four exist within AtUBC14. The introns in common interrupt the coding regions at identical positions and contain the consensus GT/AG intron borders, with the exception of the 5'-most intron of AtUBC7, which contained GC/AG borders (data not shown). AtUBC7/13/14 do not contain introns outside of the coding region, at least within the region corresponding to the cDNAs.

DNA gel blots of Arabidopsis DNA digested with a variety of restriction endonucleases revealed multiple fragments that hybridized to a coding region probe from AtUBC7 (Fig. 4). For each endonuclease used, three hybridizing fragments were identified, even at reduced stringency. Similar analyses using probes unique to AtUBC7, AtUBC13, or AtUBC14 showed that each probe specifically hybridized to one of the three fragments, indicating that AtUBC7/13/14 likely constitute the entire gene family in Arabidopsis (Fig. 4).

RNA gel-blot analysis of the AtUBC7/13/14 family detected ~950-base mRNAs in all tissues examined, including leaf, flower, silique, and stem (Fig. 5A; data not shown). Immunoblot analyses using anti-ZmUBC7 immunoglobulins indicated that the corresponding protein(s) were also present in these tissues (data not shown). The great degree of nucleotide sequence homology between the AtUBC7/13/14 genes, minimal length and high A/T content of non-homologous regions, and relatively low mRNA levels precluded the use of gene-specific probes to detect individual expression patterns by RNA gel blot analysis. Consequently, the expression patterns of the three genes were analyzed individually using quantitative RT-PCR employing gene-specific oligonucleotide primers (Fig. 5B). cDNA was generated simultaneously from individual RNA pools to minimize sample variation, and PCR reactions for all primer pairs were performed simultaneously to allow comparison of the relative PCR product levels between RNA sources. To validate this method, we analyzed mRNA levels of the homoeotic gene agamous, which previously had been shown to be expressed in flowers, but not in leaves (Yanofsky et al., 1990). This was confirmed by the greater levels of agamous PCR product derived from flower and silique RNA relative to that from leaf RNA (~100-fold (Fig. 5B)). Primers specific to each of the AtUBC7/13/14 genes produced RT-PCR products of the expected size. Whereas levels of the AtUBC7 and AtUBC13 PCR products were invariant among leaf, flower, and silique tissues, increased levels of the AtUBC14 PCR product, relative to that of AtUBC7/13, were seen in leaves (Fig. 5B). We found no increase in expression of the AtUBC7/13/14 gene family following a heat stress (37 °C for 2 h) sufficient to increase Arabidopsis HSP70 and HSP100 mRNA levels (data not shown). (In yeast, the ScUBC4/5 gene family is strongly responsive to heat stress (Seufert and Jentsch, 1990). Unlikely yeast ScUBC5 and ScUBC7 (Jungmann et al., 1993), the AtUBC7/13/14 mRNAs did not accumulate following exposure of plants to levels of cadmium (10 μM) that severely inhibited growth (data not shown).

Analyses of Recombinant AtUBC7/13/14 Proteins—The biochemical properties of AtUBC7/13/14 proteins were examined...
following expression of the respective cDNAs in E. coli. Analysis by SDS-PAGE and immunoblotting revealed that the recombinant proteins migrated at the expected molecular mass of ~19 kDa and were recognized by anti-ZmUBC7 immunoglobulins (Fig. 6A; data not shown). The recombinant E2s were enzymatically active as judged by their ability to form an adduct with [125I]-labeled ubiquitin in the presence of ATP and E1 (Fig. 6B). These adducts were unstable under reducing conditions, consistent with the linkage of ubiquitin to the E2s via a thiol-ester bond (data not shown).

Like TaUBC7, the AtUBC7/13/14 E2s conjugated ubiquitin to ubiquitin in vitro, and following prolonged incubation could generate multiquitin chains containing as many as seven monomers (see Fig. 8B for the SDS-PAGE profile of chains assembled in vitro by AtUBC7). To compare accurately the kinetics of these three E2s in multiquitin chain formation, we assayed for the assembly of diubiquitin from free ubiquitin using equivalent amounts of E2 activity (as determined by thiol-ester assay) (Fig. 7). AtUBC14 was the most active (1.9 μmol of di-ubiquitin/min), followed by AtUBC7 (1.2 μmol/min) and AtUBC13 (0.7 μmol/min). These rates, however, were significantly lower than that of TaUBC7 under the same reaction conditions (3.8 μmol/min).

Production and Analyses of Transgenic Arabidopsis—To understand better the in vivo functions of the AtUBC7/13/14 E2 family, we attempted to alter the levels of AtUBC7 in transgenic Arabidopsis using either sense or antisense approaches (van der Krol et al., 1990; Matzke and Matzke, 1995). If the AtUBC7/13/14 family is a limiting factor in multiquitin chain synthesis, we expected that an increase in active AtUBC7 would subsequently increase the ratio of multiquitin chains to ubiquitin monomers whereas a decrease in active AtUBC7 would decrease the ratio. The coding region from the AtUBC7 cDNA was placed in the forward and reverse orientations relative to the cauliflower mosaic virus 35 S promoter and nopaline synthase transcriptional terminator and introduced into Arabidopsis cv. C24. The cauliflower mosaic virus 35 S promoter affords high level, constitutive expression of many genes in plant tissues (Jefferson et al., 1987), whereas the nopaline synthase terminator effectively terminates transcription by plant RNA polymerases (Benfey et al., 1990). We obtained six independent transgenic lines expressing levels of antisense AtUBC7 mRNA that far exceeded the levels of the wild-type AtUBC7 13/14 mRNA (data not shown). However, the amount of AtUBC7/13/14 protein in each of these lines was not noticeably altered, and the transgenic plants were phenotypically normal by all parameters assayed. These included sensitivity to prolonged drought, water stress, heat stress, light stress, days and leaf number before flowering under short and long photoperiods, and fresh weight at time of flowering (data not shown).

With the sense AtUBC7 vector, we obtained 11 independent lines producing increased levels of AtUBC7 mRNA and protein (Fig. 8A and data not shown). None of these lines exhibited co-suppression of the wild-type AtUBC7 13/14 genes which can occur when endogenous genes are ectopically expressed in Arabidopsis (Matzke and Matzke, 1995). AtUBC7 protein from these lines comigrated with AtUBC7 produced in E. coli and was recognized by anti-ZmUBC7 immunoglobulins (Fig. 8A). Quantitative immunoblot analysis using purified, recombinant AtUBC7 as the standard indicated that plants homozygous for the introduced DNA accumulated ~7-fold more AtUBC7 protein than non-transformed plants. This increase was evident in almost all tissues examined, including roots, stems, leaves, inflorescence meristems, flowers, siliques, and etiolated seedlings (Fig. 8A; data not shown). The only exception was seeds...
where little accumulation of AtUBC7 was detected.

To demonstrate that the AtUBC7 protein expressed in plants was enzymatically active, we partially purified AtUBC7 from transgenic leaf tissue and examined its ability to form a thiol-ester adduct with $^{125}$I-labeled ubiquitin. Whereas the amount of adduct formed by endogenous AtUBC7 in non-transformed plants was below our limits of detection, in transgenic lines producing high levels of AtUBC7 protein we detected a ubiquitinated product that comigrated with authentic AtUBC7-ubiquitin thiol-ester adduct (Fig. 8C). The specific activity of AtUBC7 expressed in the transgenic plants was equal to, or slightly greater than, recombinant AtUBC7 produced in E. coli, as determined by quantitative thiol-ester assays (data not shown).

To test the hypothesis that AtUBC7/13/14 function in vivo to assemble multiubiquitin chains, we compared the ubiquitin conjugate profiles in cell lysates from non-transformed plants to those from AtUBC7-overexpressing plants by SDS-PAGE and immunoblotting. In wild-type Arabidopsis extracts, free multiubiquitin chains are the most abundant ubiquitin conjugates, easily detected as an immunoreactive ladder differing by $\sim 8$-kDa increments (van Nocker and Vierstra, 1993). As seen in Fig. 8B, free multiubiquitin chains containing from 2 to as many as 5 monomers could be detected that comigrated with authentic Gly$^{76} \rightarrow$ Lys$^{48}$ chains synthesized in vitro using AtUBC7. The chains were abundant in all tissues examined, including root, seed, leaf, stem, flower, and etiolated seedling (Fig. 8B). Surprisingly, in AtUBC7 overexpressing plants, no change was apparent in either the abundance or distribution of free multiubiquitin chains or in the abundance of other ubiquitinated proteins relative to that of the free ubiquitin monomers (Fig. 8B). Transgenic plants producing high levels of active AtUBC7 protein also were phenotypically indistinguishable from non-transgenic plants by all assayed parameters (see above).

**DISCUSSION**

Multiubiquitin chains linked through Gly$^{76} \rightarrow$ Lys$^{48}$ function as a strong signal in directing proteolysis when attached to various target proteins (Chau et al., 1989; Ciechanover, 1994). Despite its importance to basic cell biology, the mechanism by which target proteins become multiubiquitinated is not yet known. We have previously reported the isolation and molecular characterization of TaUBC7, a ubiquitin-conjugating enzyme from wheat capable of forming multiubiquitin chains linked exclusively through Gly$^{76} \rightarrow$ Lys$^{48}$ from free ubiquitin in vitro (van Nocker and Vierstra, 1991). Here, we report the isolation and characterization of both cDNA and genomic clones comprising a family of three transcribed genes from Arabidopsis, designated AtUBC7, -13, and -14, encoding structural homologs of TaUBC7 that are also capable of multiubiquitin chain formation in vitro. All three recombinant Arabidopsis E2s exhibited slightly different kinetics in this reaction that were significantly lower than that of TaUBC7. However, because our studies employed a recombinant wheat E1, the lower rate of chain formation by the Arabidopsis E2s compared with TaUBC7 may reflect a preference for interacting with a more closely related E1.

DNA gel blot analysis under conditions of low stringency and extensive screenings of both genomic and cDNA expression libraries indicate that AtUBC7/13/14 constitute the entire gene family in Arabidopsis. This genomic organization is typical of that seen for other Arabidopsis E2s; the AtUBC1 and AtUBC4 families contain three members each (Sullivan et al., 1994), and the AtUBC8 family contains at least five members (Girod et al., 1993). Chromatographic analyses of E2s from wheat suggest that multiple isoforms of TaUBC7 likely exist in this species as well, but whether they are encoded by different genes has not been determined (van Nocker and Vierstra, 1991).

The AtUBC7/13/14 cDNAs were predicted to be nearly full-length by comparison with the mRNA lengths of $\sim 950$ bases. For each cDNA isolated, heterogeneity was observed in the length of both the 5' and 3' non-translated regions. Some, or all, of the variability in 3' sequence length was an artifact of library construction. However, at least for AtUBC7, the presence of two poly(A) addition sites was evident. Both RNA gel blot analysis and RT-PCR indicate that AtUBC7/13/14 are actively transcribed in most, if not all, Arabidopsis tissues. In leaves, flowers, siliques, and stems, AtUBC7/13/14 mRNAs accumulate to approximately the same abundance and are present at the same ratio, with the exception of AtUBC14, which is relatively more abundant in leaves. The AtUBC7/13/14 genes are also expressed in etiolated seedlings, as this was the source of the tissue from which the cDNA libraries were created. The wide tissue distribution of AtUBC7/13/14 proteins indicate that this family of E2s has a pervasive role in basic cellular functions. The corresponding mRNAs do not accumulate in response to heat stress or cadmium exposure. This data suggest that the corresponding E2s are either not involved in the response to these stresses, or are already at sufficient levels to carry out a stress-related function.

AtUBC7/13/14 encode acidic, $\sim 19$-kDa proteins of $166\sim167$ amino acids with $76\%$ sequence identity to wheat TaUBC7 and $50\%$ identity to yeast ScUBC7. They contain the $\sim 150$-amino-
acid core domain identified in all E2s characterized to date that includes an active-site cysteine for ubiquitin transfer. In AtUBC7/13/14, the core domain is interrupted by a short (−13 amino acids) insertion near the active site. Among 27 other E2s characterized to date at the molecular level, similar internal insertions are found only in yeast ScUBC3 (CDC34) and ScUBC7, and TaUBC from wheat (Goebl et al., 1988; van Nocker and Vierstra, 1991; Jungmann et al., 1993). Comparison of the amino acid sequence of these proteins with that of AtUBC1 and yeast ScUBC4, whose crystallographic structures have been determined (Cook et al., 1993), suggests that this internal sequence extends outward as a loop from the surface of the protein and may not be involved in intramolecular interactions. This loop may provide the necessary binding site for ubiquitin recognition during chain formation. AtUBC7/13/14 proteins show the greatest structural dissimilarity to yeast ScUBC7 and wheat TaUBC7 at the amino-terminal region. This amino-terminal heterogeneity is typical of that found among E2s from Arabidopsis and other organisms. Based on the crystal structure of AtUBC1 and ScUBC4, the extreme amino terminus likely extends away from the core of the protein (Cook et al., 1993) and may interact with E1 based on amino-terminal deletion analyses (Sullivan and Vierstra, 1991).

Given the strong amino acid conservation between the AtUBC7/13/14 proteins and ScUBC7, it is tempting to speculate that these proteins are functionally analogous, in spite of the fact that AtUBC7/13/14 mRNAs do not accumulate following exposure of the plant to cadmium. However, inferring function based on structural conservation is complicated by the presence of at least one other yeast E2 exhibiting homology to AtUBC7/13/14, the cell cycle regulator CDC34. Although CDC34 differs in overall structure (containing a negatively charged domain appended to the carboxyl terminus of the core), within the core region CDC34 exhibits as great an amino acid sequence similarity (68%) to AtUBC7 as does ScUBC7 (66%).

Ectopic expression of AtUBC7 mRNA in transgenic Arabidopsis resulted in the accumulation of high levels of active protein in most tissues examined. However, no change in ubiquitin conjugate profiles were seen, nor were any phenotypic effects evident. We offer several explanations to account for this. First, AtUBC7 may function in vivo in capacities other than in multiquitin chain formation. Second, it is possible that the levels of AtUBC7 protein through high level expression of antisense mRNAs. An alternative approach would be to interfere with the function of AtUBC7/13/14 E2s by expressing structurally altered forms. A particularly attractive possibility in this regard would be a Cys→Ser substitution at the active site. This substitution in yeast RAD6 and AtUBC1 still allows ubiquitin to bind to the E2s, in this case through an ester linkage, but precludes transfer of the bound ubiquitin to substrates (Sung et al., 1990; Sullivan and Vierstra, 1993). Baillie et al. (1994) have shown that such inactive E2s can act in a dominant negative manner when expressed to high levels in yeast.

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REFERENCES

Bailly, V., Lamb, J., Sung, P., Prakash, S., and Prakash, L. (1994) Genes & Dev. 8, 1050–1060
Bartling, D., Rehling, P., and Weiler, E. (1993) Plant Mol. Biol. 23, 387–396
Beers, E. B., Moreno, T. N., and Callis, J. (1992) J. Biol. Chem. 267, 15432–15439
Benfey, P. N., Ren, R., and Chua, N. H. (1990) EMBO J. 9, 1677–1684
Callis, J. A., Raasch, J. A., and Vierstra, R. D. (1990) J. Biol. Chem. 265, 12486–12493
Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Eck, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1583
Chen, P., Johnson, P., Sonner, T., Jentsch, S., and Hochstrasser, M. (1993) Cell 74, 357–369
Chen, P., and Pickart, C. M. (1990) J. Biol. Chem. 265, 21835–21842
Chen, Z., Niles, E. G., and Pickart, C. M. (1991) J. Biol. Chem. 266, 1598–1607
Clechanova, A. (1994) Cell 79, 13–21
Cook, W. J., Jeffrey, L. C., Xu, Y. and Chau, V. (1993) Biochemistry 32, 13811–13817
Dean, C., Tamki, S., Dunsuir, P., Favreau, M., Kutayama, C., Dooner, H. and Bedbrook, J. (1986) Nucl. Acid. Res. 14, 2229–2240
Drayson, J., Haerlier, P., and Smithies, O. (1984) Nucl. Acid. Res. 12, 387–395
Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061
Felbinger, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
Genschik, P., Durr, A., and Fleck, J. (1994) Mol. & Gen. Genet. 244, 548–556
Girod, P.-A., Carpenter, T. B., van Nocker, S., Sullivan, M. L., and Vierstra, R. D. (1993) Plant J. 3, 545–552
Glotzer, M., Murray, S. W., and Kirschner, M. W. (1991) Nature 349, 138–138
Goebl, M. G., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. (1988) Science 241, 1331–1335
Hatfield, P. M., Callis, J. L., and Vierstra, R. D. (1990) J. Biol. Chem. 265, 15813–15817
Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
Hochstrasser, M., Ellison, M. J., Chau, V., and Varshavsky, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 88, 4610–4610
Jabben, M., Shanklin, J., and Vierstra, R. D. (1989) J. Biol. Chem. 264, 4998–5005
Jerschow, A., and Ciechanover, A. (1992) J. Biol. Chem. 267, 30408–30414
Jungmann, J., Reins, H.-A., Schobert, C., and Jentsch, S. (1993) Nature 361, 369–371
Laemmli, U. K. (1970) Nature 227, 685–680
Madura, K., and Varshavsky, A. (1994) Science 265, 1454–1458
Matzke, M. A., and Matzke, A. J. M. (1995) Plant Physiol. 107, 679–685
Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 773–785
Rosenberg, A. H., Lade, B. N., Chui, D., Lin, S. W., Dunn, J. J., and Studier, F. W. (1987) Gene (Amst.) 56, 125–135
Sambrook, J., Fritsch, E. J., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

S. Davis, unpublished results.
Arabidopsis AtUBC7/14 Gene Family

Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) Cell 75, 495–505
Scheffner, M., Nuber, U., and Huibregtse, J. M. (1995) Nature 373, 81–83
Schindler, U., Menkens, A. E., Ahmad, M., Bedkmann, H., Ecker, J. R., and Cashmore, A. R. (1992) EMBO J. 11, 1261–1273
Seufert, W., and Jentsch, S. (1990) EMBO J. 9, 543–550
Sullivan, M. L., and Vierstra, R. D. (1991) J. Biol. Chem. 266, 23878–23885
Sullivan, M. L., and Vierstra, R. D. (1993) J. Biol. Chem. 268, 8777–8780
Sullivan, M. L., Carpenter, T. B., and Vierstra, R. D. (1994) Plant Mol. Biol. 24, 651–661
Sung, P., Prakash, S., and Prakash, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2695–2699
Treier, M., Stasiewski, L. M. & Bohmann, D. (1994) Cél 76, 787–798
Valvekens, D., Van Montagu, M., and Van Lusebettens, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5536–5540
van der Krol, A. R., Mur, L. A., de Lange, P., Mol, J. N., and Stuitje, A. R. (1990) Plant Mol. Biol. 14, 457–466
van Nocker, S., and Vierstra, R. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10297–10301
van Nocker, S., and Vierstra, R. D. (1993) J. Biol. Chem. 268, 24766–24773
van Nocker, S., Deveraux, Q., Rechsteiner, M., and Vierstra, R. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 856–860
Vieira, J., and Messing, J. (1987) Methods Enzymol. 153, 3–11
Vierstra, R. D. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 385–410
Yandell, M. F., Ma, H., Bowman, J., Drews, G. N., Feldman, K. A., and Meyerowitz, E. M. (1990) Nature 346, 35–39