

Molecular Characterization of Plant Ubiquitin-Conjugating Enzymes Belonging to the UbcP4/E2-C/UBCx/UbcH10 Gene Family¹

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The anaphase promoting complex or cyclosome is the ubiquitin-ligase that targets destruction box-containing proteins for proteolysis during the cell cycle. Anaphase promoting complex or cyclosome and its activator (the fizzy and fizzy-related) proteins work together with ubiquitin-conjugating enzymes (UBCs) (E2s). One class of E2s (called E2-C) seems specifically involved in cyclin B1 degradation. Although it has recently been shown that mammalian E2-C is regulated at the protein level during the cell cycle, not much is known concerning the expression of these genes. Arabidopsis encodes two genes belonging to the E2-C gene family (called *UBC19* and *UBC20*). We found that *UBC19* is able to complement fission yeast (*Schizosaccharomyces pombe*) *UbcP4-140* mutant, indicating that the plant protein can functionally replace its yeast ortholog for protein degradation during mitosis. In situ hybridization experiments were performed to study the expression of the E2-C genes in various tissues of plants. Their transcripts were always, but not exclusively, found in tissues active for cell division. Thus, the *UBC19/20* E2s may have a key function during cell cycle, but may also be involved in ubiquitylation reactions occurring during differentiation and/or in differentiated cells. Finally, we showed that a translational fusion protein between *UBC19* and green fluorescent protein localized both in the cytosol and the nucleus in stable transformed tobacco (*Nicotiana tabacum* cv Bright Yellow 2) cells.

Transition from one phase of the cell cycle to another is accomplished through changes of activity of key regulatory proteins. The correct progress through the cell cycle is thus under the control of successive events where protein activation alternates with protein degradation mediated by the ubiquitin-dependent proteolytic pathway. Degradation via this pathway is a two-step process: The protein is first tagged by the covalent attachment of polyubiquitin chain(s) and then degraded by the multicatalytic protease complex called the 26S proteasome (for reviews, see Hershko and Ciechanover, 1998; Ciechanover et al., 2000). Conjugation of ubiquitin to the protein involves a cascade of three enzymes: E1, E2, and E3. The E1 (ubiquitin-activating) enzyme forms a high-energy bond with ubiquitin, which is then transesterified to an ubiquitin-conjugating enzyme (UBC) (E2). The transfer of ubiquitin to the target protein substrate usually requires ubiquitin-ligase

activity (E3). The specificity in targeting a protein for ubiquitylation resides primarily in cognate pairs of E2 and E3 enzymes.

At the onset and during anaphase, several key proteins are degraded; among them, mitotic cyclins (for review, see Murray, 1995), the anaphase inhibitors such as Brewer's yeast (*Saccharomyces cerevisiae*) Pds1p (Cohen-Fix et al., 1996) and fission yeast (*Schizosaccharomyces pombe*) Cut2p (Funabiki et al., 1996), the mitotic spindle associated Ase1p protein (Juang et al., 1997), the DNA replication inhibitor called geminin (McGarry and Kirschner, 1998), and the chromokinesin Xkid protein involved in chromosome alignment during metaphase (Funabiki and Murray, 2000). All of these substrates carry a nine-amino acid sequence called the destruction box (Dbox) motif. Another degradation signal, called the KEN-box, has also been recently identified (Pfleger and Kirschner, 2000). The E3 activity involved in this process is located on a large complex called APC/C or cyclosome, which is regulated by phosphorylation and also requires activator proteins called Cdc20/Fizzy and Cdh1/Fizzy-related (for review, see Morgan, 1999; Page and Hieter, 1999; Peters, 1999; Zachariae and Nasmyth, 1999). Although the mechanism of the Dbox or KEN-box recognition via the activator proteins has been solved very recently

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(for review, see Vodermaier, 2001), the identity of the E2 and its function during APC-dependent ubiquitylation is still poorly understood.

One E2 responsible for ubiquitylation of cyclin B, has been enzymatically purified and identified as E2-C in clam egg extract (Hershko et al., 1994; Sudakin et al., 1995; Aristarkhov et al., 1996) and as UBC-x in *Xenopus* sp. egg extracts (King et al., 1995; Yu et al., 1996). A fission yeast gene called *UbcP4*, which is involved in Cdc13 cyclin degradation and is essential for entry into anaphase, has also been identified (Osaka et al., 1997). Finally, the human ortholog of E2-C, named UbcH10, has been characterized (Townsend et al., 1997), and the overexpression of a dominant-negative form of this protein in transfected mammalian cells arrests the cells in mitosis. All of these proteins belong to a class of E2 constituted by the conserved core domain containing the catalytic Cys and an N-terminal extension whose function is still unclear. In budding yeast, the E2 enzyme most similar in sequence to E2-C, Ubc11p, does not carry the N-terminal extension domain and is not essential for yeast cell viability (Townsend and Ruderman, 1998). The genome sequence of *C. elegans* surprisingly did not reveal a gene structurally related to E2-C (Jones et al., 2001), indicating that *C. elegans* and budding yeast use one or other E2s for APC-mediated proteolysis. Yeast Ubc4p (another E2 member) supports cyclin Clb2p ubiquitylation in vitro, in the presence of the E1 enzyme and the APC ringH2 finger protein Apc11 (Levenson et al., 2000). *Xenopus* sp. and human orthologs of Ubc4p work also in concert with purified APC for the ubiquitylation of Dbox-containing proteins in vitro (King et al., 1995; Yu et al., 1996; Charles et al., 1998). Only human Ubc4 unexpectedly worked in in vitro ubiquitylation reactions with Apc11, but not the E2 UBC-x (Gmachl et al., 2000). Thus, although two classes of E2 (e.g. E2-C/UBC-x and UBC4) seem to be involved in the Dbox pathway, it is not yet clear how the E2s mediate APC-dependent protein ubiquitylation in vivo.

Furthermore, not much is known about the regulation of E2s expression during the cell cycle and in differentiated cells. It was shown that mammalian E2-C gene expression was up-regulated during oncogenic transformation (Arvand et al., 1998) and down-regulated in aging human fibroblasts, like several other genes involved in G2/M transition of the cell cycle (Ly et al., 2000). The work of Yamanaka et al. (2000) curiously suggested that mammalian E2-C is not regulated at the transcriptional but rather at the posttranslational level, because the protein is itself a substrate of APC/C dependent proteolysis.

Here, we describe the molecular characterization of two Arabidopsis E2s, called UBC19 and UBC20, structurally related to the UbcP4/E2-C/UBCx/UbcH10 protein family. We demonstrated the ability of the plant UBC19 to functionally complement the fission yeast *UbcP4-140* mutant and investigated

UBC19/20 expression patterns in various plant organs and tissues.

RESULTS

Arabidopsis Encodes Two Ubiquitin-Conjugating Enzymes Structurally Related to the UbcP4/E2-C/UBCx/UbcH10 Class of E2s

The sequences of the clam E2-C (Aristarkhov et al., 1996) and *Xenopus* sp. UBC-x (Yu et al., 1996) were used for Arabidopsis database searches and led to the identification of two different genes called *UBC19* and *UBC20*, located on Arabidopsis chromosome 3 (At3g20060) and chromosome 1 (At1g50490), respectively. Both genes are expressed because their corresponding expressed sequence tags can be found in the currently available databases. The sequence of the longest expressed sequence tag for *UBC19* was determined, and it encodes an 181 amino acids open reading frame which is complete at its N-terminal end as indicated by an in-frame stop codon upstream of the initiation codon. The protein sequence of UBC20 exhibits greater than 90% sequence identity to UBC19. A phylogenetic tree including all of the 37 E2s from Arabidopsis (Bachmair et al., 2001), indicated that UBC19/20 are more closely related to the UbcP4/E2-C/UBCx/UbcH10 E2s than any other Arabidopsis E2 (Fig. 1A). Bootstrap values derived from the neighbor-joining method (Saitou and Nei, 1987) were all above 55% inside of each clade (data not shown), further supporting the evolutionary relationship between the different E2 subfamilies. Both Arabidopsis UBC19 and UBC20 amino acid sequences were aligned with human, mouse, Goldfish, *Xenopus* sp., clam, and fission yeast E2-C orthologs and also Brewer's yeast UBC11 (Fig. 1B). The Arabidopsis E2 sequences contain the short N-terminal extension that distinguishes this subgroup from other E2s, with the exception of yeast UBC11. The plant proteins carry even the longest N-terminal extensions (36 amino acids for UBC19, compared with the 30 amino acid extensions in mammals, *Xenopus* sp., and clam).

The crystal structure of clam E2-C has recently been solved (Jiang and Basavappa, 1999). The authors showed that most of the residues that are specifically conserved into this E2 class of enzymes contribute rather to the protein surface, suggesting that they are at the molecular interface for protein interaction and specificity. Most of these residues are conserved in the plant E2 protein sequences (residues V31, Q36, M44, F53, D55, T65, D108, T130, G140, L148, N158, K163, and Y170; the identity and number of the residues is based on clam sequence).

UBC19 Protein Forms a Thiol-Ester Bond with Ubiquitin in Vitro

In E2s, the active site Cys forms a thiol-ester linkage with the C-terminal carboxyl of ubiquitin. The

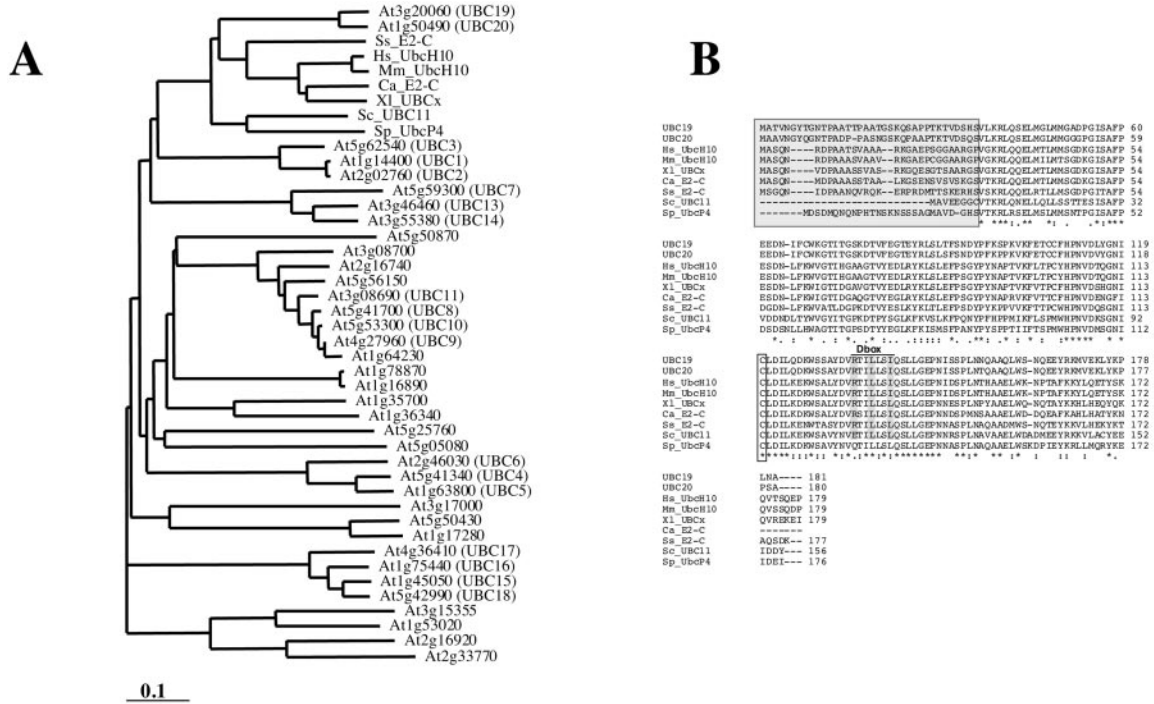


Figure 1. Phylogenetic tree and sequence alignments of the E2-C proteins. A, Phylogenetic tree performed with the Clustal program including all Arabidopsis E2s and the E2s from other organisms belonging to the E2-C class. Branch lengths are proportional to phylogenetic distances. B, Alignment of the Arabidopsis UBC19 and UBC20 amino acid sequences with those of human UbcH10, mouse UbcH10, *Xenopus* sp. UbcX, goldfish E2-C, clam E2-C, Brewer's yeast Ubc11p, and fission yeast UbcP4. Multiple sequence alignment was performed with ClustalW (1.81) program. The N-terminal extension domains and the active Cys residues are boxed. The localization of the Dbox sequence is indicated. Asterisks and dots indicate identical and conserved amino acids, respectively. UBC19 and UBC20 nucleotide and amino acid sequences have been submitted to the EMBL/GenBank sequence libraries under accession numbers AY127573 and AY127574, respectively.

coding region of Arabidopsis UBC19 cDNA was cloned in the pET11d-inducible expression vector, and the enzyme was overexpressed in *Escherichia coli*. A mutant version of the protein, in which the catalytic Cys was replaced by a Ser residue, was also produced in *E. coli*. Bacterial protein extracts expressing either the wild type or the mutant protein were assayed in vitro for ester formation of the recombinant polypeptides with radiolabeled-ubiquitin in the presence of Arabidopsis ubiquitin-activating enzyme E1 (UBA1; Hatfield et al., 1997) and ATP (Fig. 2). As a control, we used purified Arabidopsis UBC7 protein. In the presence of the bacterial extracts, a radiolabeled protein complex with a size of around 36 kD could be detected (Fig. 2, lanes C and D). As expected, the complexes formed between the native UBC19 (or UBC7) and ubiquitin were sensitive to boiling under reducing conditions, indicating that the proteins are linked via a thiol-ester bond (Fig. 2, +DTT, lanes C and E). The protein complex with the mutant protein UBC19 (C/S) was resistant to boiling in the presence of DTT, most likely because of the formation of an oxygen-ester (lane D). Finally a mutant version of the protein, in which the catalytic Cys was replaced by an Ala residue, UBC19 (C/A), was unable to form a complex with ubiquitin (data not shown).

UBC19 Complements Fission Yeast Mutant UbcP4-140

Disruption of the fission yeast gene *UbcP4* produces both a G2/M and a metaphase/anaphase cell cycle arrest (Osaka et al., 1997; Mitsuzawa et al., 2001). We tested whether the plant protein is able to rescue the fission yeast *ts* mutant *UbcP4-140* (Mitsuzawa et al., 2001). For the complementation experiments, different constructs were designed (see Fig. 3A): the full-length UBC19, a dominant negative version of UBC19 (C/S) and a chimeric gene, in which the N-terminal extension domain of UBC19 was replaced by the N-terminal extension domain of yeast UbcP4. The different constructs were put under the control of the thiamine-repressible promoter *pnmt1* of the *pREP1* expression vector and transformed into *UbcP4-140* cells. At nonpermissive temperature and in the absence of thiamine, both the plant E2 and the chimeric E2 allowed the growth of *UbcP4-140* mutant (Fig. 3B), whereas another Arabidopsis E2 UBC9 (At4g27960) belonging to a different E2 subfamily was unable to complement the mutant strain (data not shown). Interestingly, the chimeric protein [UbcP4(Nt)/UBC19] was more efficient for the growth rescue of the yeast mutant strain than the wild-type plant E2 (data not shown), suggesting that the N-terminal extension may carry important struc-

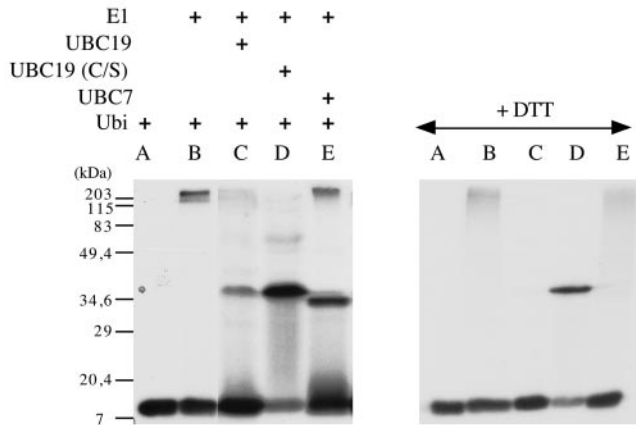


Figure 2. Thioester complex formation between ubiquitin and the UBC19 protein. Assay reactions contained ATP, ^{32}P -labeled ubiquitin overexpressed in *E. coli*, purified Arabidopsis E1 enzyme UBA1, and crude extracts from bacteria expressing UBC19 (lane C), UBC19 (C/S; lane D), or UBC7 (lane E) protein. Reactions were analyzed by SDS-PAGE under nonreducing or reducing (+dithiothreitol [DTT]) conditions, and radiolabeled proteins were visualized by autoradiography. Control reactions containing labeled ubiquitin incubated with non-transformed bacterial extracts with (lane B) or without (lane A) the E1 enzyme are shown. Extracts from bacteria expressing UBC19 incubated with ^{32}P -labeled ubiquitin but without the E1 enzyme were unable to form the thioester complex (data not shown).

tural information involved in the E2 function. As expected, UBC19 (C/S) worked as a dominant negative mutant protein because it inhibited the growth of *UbcP4-140* mutant strain already at permissive temperature. If this strain was grown at permissive temperature but in the presence of thiamine, which represses UBC19 (C/S) expression, no growth defect was detected (data not shown).

Southern-Blot Analysis

To investigate the expression patterns of both genes by RNA gel and in situ hybridization, we tested first the specificity and cross-reactivity of the cDNA probes. Genomic DNA from Arabidopsis and the closely related radish (*Raphanus sativus* cv Radis rubine scarlate) plant was digested by different restriction enzymes and hybridized with both UBC19 and UBC20 full-length cDNA probes (Fig. 4). In both Arabidopsis and radish, only weak cross-reactivity of the probes was detected. Furthermore the UBC19/20 cDNA probes did not cross-react with other E2 genes, like those belonging to the Ubc4/5 gene family (data not shown).

UBC19 and UBC20 mRNAs Accumulate in All Arabidopsis Organs and to a Higher Extent in Dividing Cells

Both *UBC19* and *UBC20* mRNAs were found to accumulate in various Arabidopsis organs and in germinating seeds (Fig. 5A). Expression of both

genes was similar but not identical. Both *UBC19* and *UBC20* mRNAs accumulate in floral buds, open flowers, stems, and roots and at low levels in siliques. Nevertheless, *UBC19* is expressed at higher level in mature leaves than *UBC20*. We also tested *UBC19* gene expression under different growth conditions including heat shock, dark to light transition and drug treatments inducing a stress response (like the proteasome inhibitor MG132 or geldanamycin); none of these experimental conditions changed its expression level (data not shown).

To study the E2 gene expression during reinitiation of mitotic activity in Arabidopsis, we used an experimental system (Genschik et al., 1994) in which differentiated leaf cells are stimulated to re-enter the cell cycle by wounding and auxin stimulation. Both *UBC19* and *UBC20* mRNAs accumulate in the leaf strips culture at the highest levels at 48 to 72 h (Fig. 5B), a time when the first cell divisions are observed (data not shown). Histone H4 mRNAs accumulate at the highest level earlier at 24 to 48 h, when most of the cells in the culture are in S phase (24 to 48 h of culture corresponding to the highest ^3H -dTTP incorporation; data not shown).

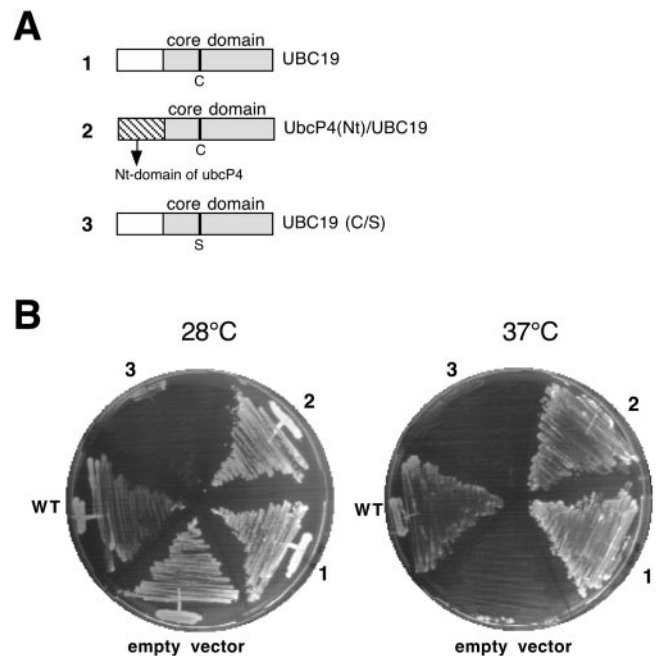
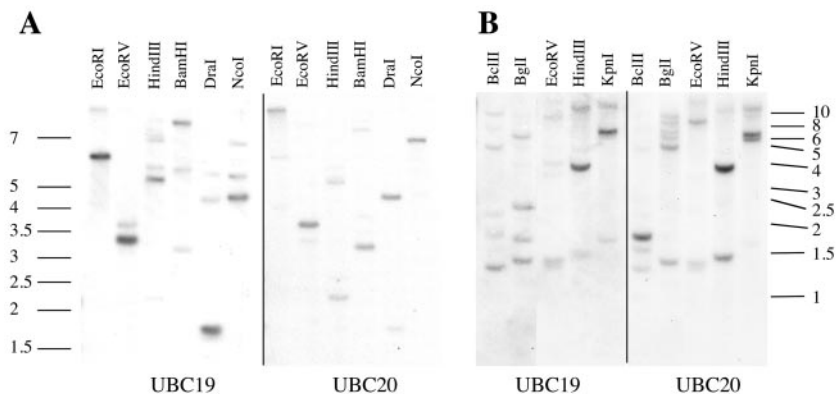


Figure 3. Functional complementation of fission yeast temperature sensitive *UbcP4-140* strain. A, Schematic representation of the constructs used for yeast complementation. All constructs were put into the pREP1 vector. Open and hatched boxes represent the N-terminal extension domains of UBC19 and Sp_UbcP4, respectively. The gray boxes represent UBC19 core domain. The catalytic Cys and the Ser (in the dominant negative version of the protein) residues are also represented. B, The yeast mutant strain was transformed with either an empty pREP1 vector or with the pRep1 vector expressing wild-type UBC19 (1) or chimeric UbcP4(Nt)/UBC19 (2) or the dominant negative UBC19 (C/S; 3). The wild-type yeast strain (WT) and the transformed *UbcP4-140* strains were grown either at permissive (28°C) or restrictive (37°C) temperature.

Figure 4. Genomic Southern-blot analysis. Five micrograms of Arabidopsis (A) or 7 μ g of radish (B) DNA was digested with the indicated restriction enzymes before electrophoresis in an agarose gel and subsequent transfer to a nylon membrane. The same membranes were successively probed with UBC19 (left panels) and UBC20 (right panels) cDNA probes. Size markers are indicated in kilobase pairs.



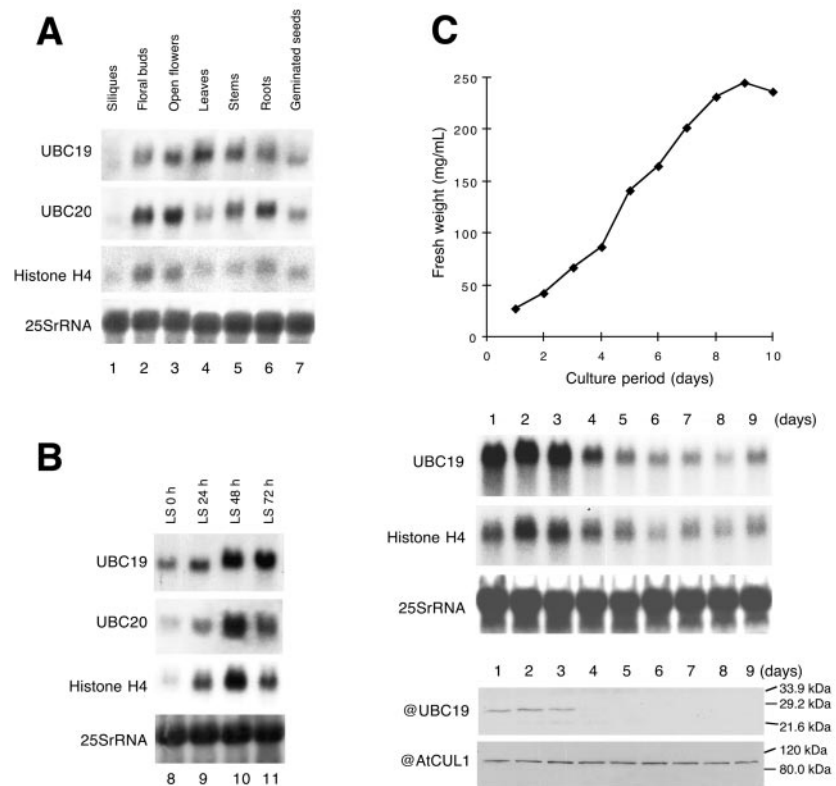
We also followed *UBC19* mRNA and protein levels during Arabidopsis cell-suspension culture (Fig. 5C). Cells rapidly entered the logarithmic growth phase and reached the stationary stage after 7 d. Both histone H4 and *UBC19* mRNAs reached their highest level of accumulation from d 1 to 3 (corresponding to the time where the highest number of cell division can be observed; data not shown). A polyclonal antibody was raised in rabbits against the 20-amino acid N-terminal peptide of UBC19 and used in western-blot analysis. The UBC19 protein was only detectable during the first days of culture corresponding to the highest level of its transcript accumulation. As a control, we used an antibody raised against AtCUL1, whose gene is constitutively ex-

pressed in Arabidopsis cell suspension culture (Shen et al., 2002).

UBC19 and Ubc20 Genes Are Expressed in Various Arabidopsis and Radish Tissues

To analyze more precisely and identify the tissues in which the plant E2s are expressed, we performed in situ hybridization using [³⁵S] UTP-labeled *UBC19* and *UBC20* anti-sense or sense probes on different sections of Arabidopsis and of its very closely related radish species (Fig. 6). Radish organs are larger than Arabidopsis and were therefore used to obtain more accurate and detailed mRNA localization. In general, both *UBC19* and *UBC20* probes revealed almost iden-

Figure 5. UBC19 and *UBC20* mRNA accumulation in various Arabidopsis organs, during reinitiation of mitotic activity in leaf strip cultures and in a cell-suspension culture. A, Total RNA was extracted from silique (lane 1), floral buds (lane 2), open flowers (lane 3), leaves (lane 4), stems (lane 5), roots (lane 6), and 3-d-old germinating seeds (lane 7); B, total RNA was extracted from leaf strips (LS) incubated in a medium suitable for cell division for 0 h (lane 8), 24 h (lane 9), 48 h (lane 10), and 72 h (lane 11). The blots were probed successively with UBC19, UBC20, histone H4, and 25S rRNA probes. C, Growth curve of the cell-suspension culture determined by the fresh weight (top right panel). Northern analysis from total RNA extracted at different times during the culture (middle right panel). The RNA blot was probed with UBC19, histone H4, and 25S rRNA probes. UBC19 protein accumulation at different times during the culture (lower right panel). The western blot was probed successively with anti-UBC19 and anti-AtCUL1 polyclonal antibodies.



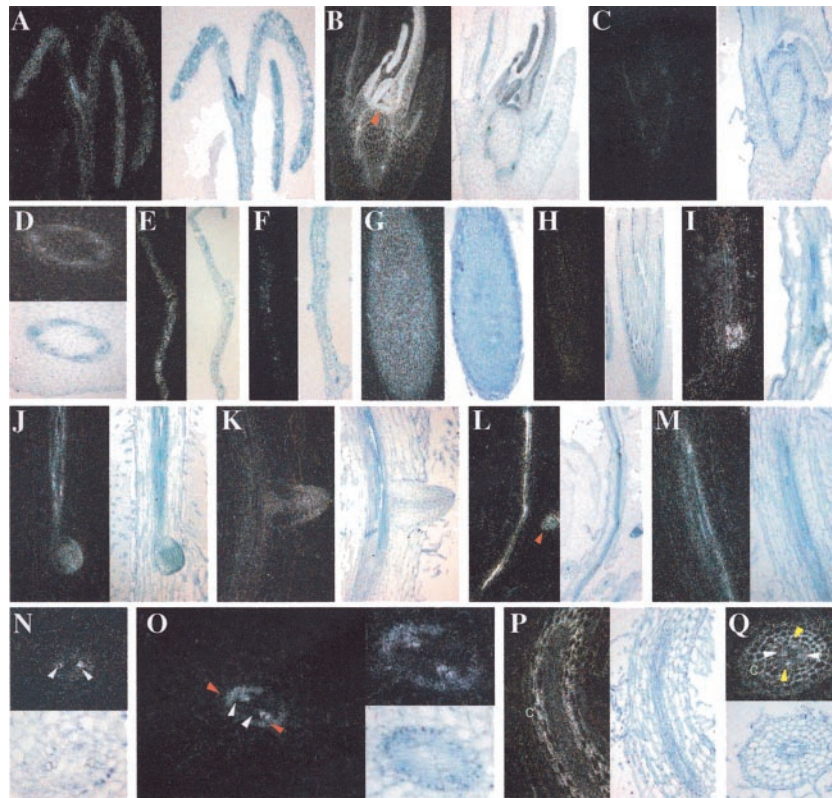


Figure 6. In situ localization of *UBC19* and *UBC20* mRNAs in various Arabidopsis and radish organs. Hybridization signals visualized as white grains in dark field optics are shown on left panels, whereas bright field images showing tissue morphology are shown on right panels. In situ hybridization with control sense probes for both E2s were systematically performed with sections of different organs analyzed and gave only very weak background signals (only shown for shoot and root apices). A, Longitudinal sections through Arabidopsis seedlings 5 d after germination shows *UBC19* expression in shoot meristem and young leaves. B, Longitudinal section through radish shoot apex shows expression of *UBC19* in the shoot apical meristem and young leaves. Arrowhead points to the shoot apical meristem. C, Longitudinal section through a shoot apex of radish hybridized with *UBC19* sense probe. D, Transverse section of a radish stem shows expression of *UBC19* in the vascular tissue. E and F, Longitudinal sections through differentiated Arabidopsis leaves hybridized with *UBC19* (E) and *UBC20* (F) anti-sense probes. G, Longitudinal section through radish root apex illustrating homogeneous expression of *UBC19* in root meristems. H, Longitudinal section through radish root apex with *UBC19* sense probe. I, Longitudinal section through an Arabidopsis root showing *UBC19* expression in emerging lateral root meristem. J and K, Longitudinal sections through radish roots showing *UBC19* expression in emerging (J) and in emerged (K) lateral root meristems. L and M, Longitudinal sections of Arabidopsis (L) and radish (M) roots showing *UBC19* transcript localization in the vascular cylinder. N, Transverse section of radish roots showing high expression of *UBC19* in protoxylem elements (white arrowheads). O, Transverse sections of differentiating radish root further away from the root tip than sections shown in N illustrating *UBC19* expression in pericycle cells at the xylem poles (red arrowheads) and xylem elements (white arrowheads). P and Q, Longitudinal (P) and transverse (Q) sections of radish roots showing *UBC20* expression in protoxylem elements (white arrowheads). Expression in phloem (yellow arrowheads) and cortex tissue (c) is only observed with *UBC20* anti-sense probe.

tical expression patterns, except in root tissues (see below). Thus, in situ hybridization with the *UBC20* probe is only shown when different from *UBC19*.

UBC19/20 genes are expressed in the shoot apical meristem, leaf primordial, and young leaves (Fig. 6, A and B). Sense probes never allowed detection of a signal either with Arabidopsis or radish sections, as illustrated with the *UBC19* sense probe (Fig. 6C). In stems, transcript localization of both E2s is seen in the vascular tissue (shown for *UBC19* in Fig. 6D). According to the RNA gel analysis (see Fig. 5A), *UBC19* is expressed at higher levels in mature leaves

than *UBC20* (Fig. 6, E and F). *UBC19/20* genes are also expressed in the root meristem and in emerging lateral roots (Fig. 6, G and I–L). Hybridization performed with sense probes gave only weak background signals, as illustrated in Figure 6H.

However, whereas *UBC19* is only expressed in the vascular cylinder (Fig. 6, J, L, and M), mainly in protoxylem elements (Fig. 6, M and N), *UBC20* probe allowed also the detection of a signal in differentiated root cortex, xylem, and phloem tissues (Fig. 6, P and Q). Furthermore both genes were expressed in pericycle cells in maturing roots at the xylem poles,

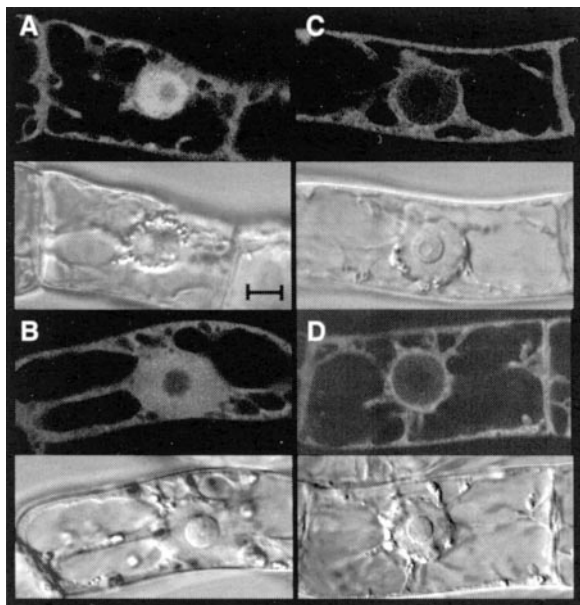


Figure 7. Subcellular localization of UBC19 using GFP as life marker in tobacco BY2 cells. Each fluorescent focal plane (top panels) is shown with its corresponding transmitted light reference image viewed by differential interference contrast (bottom panels). Localization of GFP alone (A), UBC19-GFP (B), GUS-GFP (C), and UBC19-GUS-GFP (D) in interphase BY2 cells after 24 h of Dex induction. Bar = 10 μ m.

where potentially lateral roots emerge (Fig. 6O; data not shown). Because the two E2 probes exhibit weak cross-hybridization (Fig. 4) and because the number of radish genes belonging to this E2 family is unknown, we cannot exclude that this expression pattern results from the superposition of the expression of several, still yet unknown, radish *UBC19/20* homologs. To determine to what extent this E2 gene family is subjected to differential tissue-specific expression, additional experiments are required. Differential tissue-specific expression has already been reported for the Arabidopsis *UBC4-6* gene family (Thoma et al., 1996), using promoter β -glucuronidase (GUS) fusion studies.

Thus, the *in situ* hybridization experiments revealed that in all tissues with cell division activities, *UBC19/20* expression could be detected: like the shoot and root meristems, expanding vascular tissues, and leaf primordia. However, the expression of these genes is not exclusively correlated with cell proliferation, because we found also expression in differentiated cells (in mature leaves and in root cortex).

UBC19/Green Fluorescent Protein (GFP) Fusion Protein Is Both Cytoplasmic and Nuclear

For subcellular localization studies, UBC19 was translationally fused to the GFP proteins, and stable transgenic tobacco (*Nicotiana tabacum* cv Bright Yel-

low 2 [BY2]) cells, expressing the fusion protein under the control of the Dex-inducible promoter (Aoyama and Chua, 1997), were established. The UBC19/GFP fusion protein, like GFP alone, was found both in the cytoplasm and the nucleus (Fig. 7, A and B). The size of the fusion protein is around 46 kD, which is close to the selective barrier (20–40 kD) of passive protein diffusion (for review, see Görlich and Kutay, 1999). To investigate whether UBC19 carries the capacity to translocate a reporter protein in the nucleus, we engineered a construct in which UBC19 was fused in frame to both GFP and GUS, increasing the size of the reporter protein to more than 100 kD. Nevertheless, this was not the case, and the UBC19/GFP/GUS fusion protein, like the GFP/GUS control protein, localized mainly to the cytoplasm (Fig. 7, C and D), indicating that UBC19 enters the nucleus only by passive diffusion.

DISCUSSION

It is now clear that the Dbox pathway is regulated at multiple levels during the cell cycle (for reviews, see Morgan, 1999; Page and Hieter, 1999; Zachariae and Nasmyth, 1999). Thus, the core APC/C ubiquitin ligase is itself regulated during the cell cycle by phosphorylation of several of its subunits. Furthermore, it has been shown that APC/C activity requires activator proteins, called Cdc20/Fizzy and Cdh1/Fizzy-related. The first class of proteins is regulated both at the transcriptional and posttranscriptional (e.g. proteolysis) levels, whereas the later is mainly regulated by phosphorylation. Nevertheless, not much is known concerning the contribution of the E2 enzymes to the APC/C activity. Two classes of E2s (E2-C/Ubc-x and Ubc4), capable of contributing to APC activities have been reported (see the introduction). Although both classes of E2s were able to work with purified APC/C *in vitro*, *in vivo* functional data are only available for the E2-C class of proteins. In fission yeast, the mutation of the gene (called *UbcP4*) produced cell cycle arrests (at G2/M and at metaphase-to-anaphase transition) and inhibited mitotic cyclin Cdc13 degradation (Osaka et al., 1997). In animal cells, overexpression in COS cells of a dominant negative version of the human E2-C protein (UbcH10) inhibited cyclin A and B1 degradation, whereas a dominant negative clam E2-C protein inhibited the onset of anaphase in frog embryonic cells (Townsend et al., 1997). Specificity of the E2 involved in mitotic events was assayed by microinjecting PtK1 cells with different dominant negative E2s (Bastians et al., 1999). These experiments clearly demonstrated that only dominant negative human UbcH10 and not UbcH5b, caused a dramatic delay in anaphase onset, suggesting that E2-C is selectively required for anaphase inhibitor degradation.

Nevertheless, how these E2s are regulated during the cell cycle and in differentiated cells is poorly

understood. Whereas the fission yeast UbcP4 protein seems to accumulate constitutively during the cell cycle (Osaka et al., 1997), the mammalian E2-C is mainly regulated at the posttranslational level by APC/C-dependent proteolysis of the protein (Yamanaka et al., 2000). RNA gel-blot analysis revealed that the goldfish E2-C mRNAs accumulate strongly in ovaries similarly to cyclin B mRNAs (Tokumoto et al., 1999).

Arabidopsis, whose genome sequencing has been achieved (The Arabidopsis Genome Initiative, 2000), encodes thirty-seven potential different E2 genes (for review, see Bachmair et al., 2001). The E2 class, to which budding yeast Ubc4 belongs, is represented in Arabidopsis by a multigenic family of eight members, whereas for the E2-C class of E2s, only two genes could be identified (*UBC19* and *UBC20*). We demonstrated that at least one of the plant genes could replace the fission yeast *UbcP4* gene for its cell cycle functions. Thus, the plant proteins are both structurally and functionally related to the E2-C class of enzymes.

Expression analyses, in Arabidopsis and radish, were performed both by RNA gels and in situ hybridization. Both *UBC19* and *UBC20* genes are more strongly expressed in tissues active for cell division, similarly to cyclin B1 (Ferreira et al., 1994; Segers et al., 1996). Interestingly, Arabidopsis *UBC19/20* promoter sequences contain a common cis-acting element, called the M-phase-specific activator element (data not shown), which is necessary and sufficient for G2/M specific gene expression (for review, see Ito, 2000). Nevertheless, whether *UBC19/20* expression occurs at a particular stage of the cell cycle remains to be demonstrated. Furthermore, it is worth to note that a strong expression in Arabidopsis meristematic regions has also been reported for E1 and other E2 genes (Thoma et al., 1996; Hatfield et al., 1997), suggesting a particular need of ubiquitylation during cell proliferation.

Although *UBC19/20* expression was always detected in tissues where cell division occurs, we also found some expression in differentiated tissues. Thus, a higher level *UBC19* mRNAs accumulated in mature leaves, whereas expression of *UBC19/20* radish homologs was detected in differentiating and mature cortex. In animal cells, it was shown that Dbox pathway is active in in vitro differentiated myoblast cells (Brandeis and Hunt, 1996) and in terminally differentiated neurons (Gieffers et al., 1999). By using a chimeric fusion protein (the N-terminal domain of cyclin A3 fused to the GUS reporter protein), we found that the Dbox pathway is active in several differentiated tobacco tissues (A. Derevier, M.C. Criqui, and P. Genschik, unpublished data). Substrates for APC/C-dependent ubiquitylation in differentiated cells are unknown. Nevertheless, this raises the interesting possibility that APC/C dependent protein degradation occurs outside of the cell

cycle, as already suggested by J.-M. Peters and collaborators (Gieffers et al., 1999) and that *UBC19/20* and their orthologs may contribute to this proteolytic pathway.

MATERIALS AND METHODS

Unless stated otherwise, all procedures for manipulating DNA and RNA were carried out according to Sambrook et al. (1989) and Ausubel et al. (1994). Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

Chemicals

Dexamethasone (Sigma-Aldrich, St. Louis) was dissolved in ethanol and stocked at a concentration of 30 mM.

Construction of Plasmids

To construct the dominant negative pSK-*UBC19*(C/S), we mutated the Cys residue in the UBC domain, involved in thiol-ester formation with ubiquitin, by PCR, using oligonucleotide (5'-CAATATTAGCTTGACATTCTTC-3') and oligonucleotide (5'-CCATAGAGATCCACATTGG-3'), respectively. The three constructs for yeast complementation assays were cloned as *Sall*-*Bam*HI fragments into the pREP expression vector (Maundrell, 1993). These restriction sites were introduced for *UBC19* and *UBC19* (C/S) by PCR amplification using oligonucleotide 5'-AGGCGAGTCGACATGGCGACGGTTAATGGGTAC-3' and oligonucleotide 5'-CAGTGATTTGGATCCTCATGCGTTAAAGGCT-3'. The chimeric UbcP4/*UBC19* fragment has been generated with the Exsite PCR site-directed mutagenesis kit (Stratagene, La Jolla, CA) with oligonucleotide 5'-AATCTAAAAAAGTCTTCTTCTGCTGGCATGGCTGTTGACGGTCATAGCGTTCTAAAAAGGTCGAATCTGAAC-3' and oligonucleotide 5'-AGTATGAGGGTCTGGTTTTGCATATCAGAATCCATCCCTTTTCGCCTTCTAATTG-3'. pTA-GFP construct is described in Criqui et al. (2000). The GFP sequence used in all experiments is the ultra-bright version of the [Leu 64 > Phe] and [Ser 65 > Thr] (see Menand et al., 1998). A *Xho*I-*Nco*I fragment of *UBC19* obtained by PCR amplification using oligonucleotide 5'-CATTAACTCGAGCCATGGCGACGGTTAATGGGTACAG-3' and oligonucleotide 5'-AATTAACCATGGATGCGTTAAAGGCTTG-3' was introduced in frame at the N terminus of GFP and resulted in plasmid pSK-*UBC19*-GFP. The *Xho*I-*Spe*I fragment was then subcloned into the dexamethasone-inducible vector pTA7002 (Aoyama and Chua, 1997) resulting in plasmid pTA-*UBC19*-GFP. In the pTA-GUS-GFP construct, the GUS coding sequence was introduced in frame with the GFP sequence after PCR amplification with oligonucleotide 5'-GATCATCTCGAGACCATGGTACGTCCTGTAGAAACCCCAA-3' and oligonucleotide 5'-ACTCATG-GATCCTTGTGGCTCCCTGCTGCGGTTT-3'. Finally, the pTA-*UBC19*-GUS-GFP construct resulted from subcloning the *UBC19* coding sequence from the pSK-*UBC19*-GFP construct into pTA-GUS-GFP vector. The PCR-amplified fragments in all cloning procedures were always sequenced on both strands.

Plant Material

The Arabidopsis plants used for the RNA gels were of the Wassilewskija ecotype. To check *UBC19* expression during the dark to light transition, Arabidopsis plants have been grown in vitro in complete darkness for 12 d, and then were transferred to standard light condition and collected after 1, 3, and 8 h. A gene encoding a photosystem II component was used as a control. For heat shock, 14-d-old Arabidopsis plants grown in vitro under standard conditions were collected and incubated in sterile water either at 38°C for the heat shock or at 22°C for the control. After 90 min of treatment, plants were incubated in sterile water at 22°C and collected 1 h later for RNA preparation. Control hybridization experiments with HSP70 and HSP105 probes, indicated a strong induction of HSP genes under our experimental conditions.

Clonal transgenic tobacco (*Nicotiana tabacum*) BY2 cell cultures for pTA-GFP, pTA-*UBC19*/GFP, pTA-GUS/GFP, and pTA-*UBC19*/GUS/GFP constructs were established. The *Agrobacterium tumefaciens*-mediated transfor-

mation protocol of the BY2 cells is described by Genschik et al. (1998). To produce the accumulation of the GFP fusion proteins into the transgenic cell lines, 5 μM dexamethasone was added to the culture medium. The handling of the BY2 cell culture were performed according to Nagata et al. (1992).

RNA and DNA Gel Blotting

RNA gels were realized with 20 μg of total RNA per lane. The RNA extraction and RNA gel-blotting procedures are described by Criqui et al. (2000). The UBC19 and UBC20 cDNA probes correspond to the complete cDNAs clones. The histone H4 probe corresponds to the 196-bp restriction fragment *AccI-DdeI* of the coding region of the gene *H4A748* (Chaboute et al., 1987). The integrity and the amount of RNA applied to each lane were verified by ethidium bromide staining and control hybridizations using an Arabidopsis 25S rRNA probe (GenBank accession no. T44938).

The genomic DNA was extracted according to (Qiagen USA, Valencia, CA). The UBC19 and UBC20 cDNA probes correspond to the complete cDNAs clones. The DNA gel blots were hybridized overnight at 42°C in 5 \times SSPE, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 1% (w/v) SDS, and 50 $\mu\text{g mL}^{-1}$ denatured salmon sperm DNA. The blots were subsequently washed in 2 \times SSC and 0.1% (v/v) SDS for 30 min at 42°C and in 0.2 \times SSC and 0.1% (v/v) SDS for 30 min at 42°C.

In Situ Hybridizations

Virtually all steps of the in situ procedure were performed essentially as described by de Almeida Engler et al. (2001). Radish (*Raphanus sativus* cv Radis rubine scarlate) and Arabidopsis (ecotype Columbia) seeds were germinated in K1 medium (Valvekens et al., 1988), and plant material was harvested for fixation. All samples were fixed in 2.5% (v/v) glutaraldehyde, dehydrated, and paraffin embedded. Sections of 10 μm thick were fixed to 3-aminopropyltriethoxy-silane-coated slides and used during the in situ hybridization procedure. UBC19 and UBC20 probes were synthesized from PCR products flanked by T3 and T7 promoters and 20 \times 10⁶ cpm slide⁻¹ were applied (10 ng slide⁻¹). Exposure times varied and images were taken by using a digital AxioCam (Zeiss, Welwyn Garden City, UK) under standard dark and bright field optics.

Overexpression of the E2 Enzymes and Ubiquitin-E2 Thiol-Ester Linkage

NcoI and *BamHI* sites were introduced 5' and 3', respectively, of pSK-UBC19 and pSK-UBC19 (C/S) by PCR-based site-directed mutagenesis. The PCR reaction was performed using oligonucleotide 5 (5'-GC-GAAAACCATGGCGACGGTTAATG-3') and oligonucleotide 2 (5'-ATTTTGTAGGATCCTCATGCGTTAAAGGCTAG-3') as the upstream and downstream primers, respectively. The *NcoI-BamHI* fragments were cloned into the pET-11d vector (Novagen, Madison, WI) to produce recombinant proteins with an additional SHHHHHH sequence at the C terminus. Overexpression was performed in *Escherichia coli* strain BL21(DE3) pLysS. The recombinant His-tagged ubiquitin containing a cAMP-dependent protein kinase phosphorylation site (Tan et al., 1999) was kindly provided by Dr. Zhen-Qiang Pan and was expressed in *E. coli* strain BL21(DE3) pLysS. The protein remained soluble and could be purified in the native form under non-denaturing conditions using the nickel-nitrilotriacetic acid agarose-based affinity purification procedures according to the protocol provided by Qiagen USA. The purified protein was further applied to a Sephadex G-25 column, equilibrated, and eluted with 50 mM NaH₂PO₄ (pH8), 300 mM NaCl, and 10 mM phenylmethylsulfonyl fluoride.

One microgram of the ubiquitin fusion protein was radiolabeled using 5 units of cAMP-dependent protein kinase (Biolabs) and 10 μCi of [γ -³²P]ATP as described by Tan et al. (1999). After the reaction the kinase was heat inactivated. For thioester assays, reactions were carried out in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM ATP, and 100 ng of ³²P-labeled ubiquitin at 30°C for 40 min. Unless stated otherwise, 500 ng of the purified Arabidopsis E1 enzyme UBA1 (Hatfield et al., 1997) and 10 μg of bacterial crude protein extracts overproducing UBC19 or its mutant proteins were used in the reaction. The protein samples (in presence or absence of DTT) were subjected to electrophoresis in a 15% (w/v) SDS-polyacrylamide gel, and autoradiography was used to visualize radiolabeled bands.

Protein Extraction, Preparation of Antibodies, and Immunoblotting

The Arabidopsis cells were homogenized using pestle and mortar in extraction buffer (25 mM Tris-HCl, pH 7.5, 15 mM MgCl₂, 15 mM EGTA, 150 mM NaCl, 0.1% [w/v] Tween 20, 1 mM DTT, and complete protease inhibitor cocktail mix [Roche Diagnostics, Indianapolis]) with PolyClar AT (Serva, Garden City Park, NY) and quartz and centrifuged at 20,000g. The protein content was determined by using the Bio-Rad protein assay kit.

Polyclonal antibodies were raised in rabbits against the N-terminal ATVNGYGTNTPAATTPAAT peptide of UBC19 protein to which an additional Cys residue at the N terminus was added for keyhole limpet hemocyanin coupling. Samples of 15 μg of proteins were separated by 15% (w/v) SDS-PAGE gels and transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were probed with the anti-UBC19 antibody diluted 1:3,000 (v/v). For protein loading control, we used a polyclonal antibody raised against Arabidopsis AtCUL1 protein (Shen et al., 2002). The antibody was used at a dilution of 1:2,500 (v/v). The immunoreactive proteins were detected using peroxidase-conjugated goat anti-rabbit antibodies (Dianova, Hamburg, Germany) and the ECL western-blot analysis system from Amersham Biosciences AB (Uppsala). Determination of the specificity of the antibodies was performed by competition experiments (data not shown). In this case, the antibody was incubated for 3 h at room temperature without or with 0.1 μM of antigen peptide before application to the membranes.

UbcP4-140 Yeast Strain Complementation

Fission yeast (*Schizosaccharomyces pombe*) UbcP4-140 *ts* mutant strain (*h-ade6-M210 leu1-32 ura4-D18 ubcP4-140::ura4*) established from strain HM530 (*h-ade6-M210 leu1-32 ura4-D18*) was kindly provided by Dr. Hiroaki Seino (Shizuoka, Japan; Mitsuzawa et al., 2001). For the manipulation of these strains, we used the conditions described by Guthrie and Fink (1991). Between three and six independent transformants per constructs were assayed in the complementation experiments that were repeated at least twice. The same results were obtained for the different experiments.

Fluorescence Imaging

Confocal images were obtained by a Zeiss LSM510 laser-scanning confocal microscope with argon laser excitation at 488 nm and through 505 to 550 emission filter-set and using a C-APOCHROMAT (63 \times 1, 2-W Korr) water objective lens. The images are presented as single sections. Transmitted light reference images were taken using differential interference contrast (Nomarski) optics and argon laser illumination at 488 nm. Images were contrast enhanced using image-processing software (Photoshop, Adobe Systems Inc., Mountain View, CA).

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