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Demonstration of ATP-Dependent, Ubiquitin-Conjugating Activities in Higher Plants[1]

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ABSTRACT

Ubiquitin is a highly conserved, 76-amino acid polypeptide with several important regulatory functions in both plants and animals that all arise from its covalent ligation to other cellular proteins. Here, we demonstrate that higher plants have the capacity to conjugate ubiquitin to other plant proteins *in vitro*. Using ^{125}I-labeled human ubiquitin as a substrate, conjugating activities were observed in crude etiolated tissue extracts from all species tested, including oats, rye, barley, corn, zucchini squash, pea, soybean, and sunflower. The reaction has a soluble distribution, is specific for ATP, and requires the protease inhibitor, leupeptin, to protect ubiquitin from inactivation during the assay. Conjugation is inhibited by *N*-ethylmaleimide and high concentrations of 2-mercaptoethanol suggesting that the mechanism of ubiquitin ligation in plants involves a similar thiolester intermediate to that found in the mammalian pathway. The conjugating activity in etiolated oat extracts is extremely labile with a half-life of about 20 minutes at 30°C. Detectable but low ATP-stimulated, conjugating activities were also observed in extracts from dry seeds and green leaves of oats. In addition to this conjugating activity, crude plant extracts have the capacity to degrade ubiquitin-protein conjugates formed *in vitro*. These results demonstrate that higher plants contain several of the enzymic activities necessary for ubiquitin's functions and provide a method for assaying ubiquitin conjugation *in vitro*.

Ubiquitin is a small (76 amino acids), abundant protein present in all eucaryotic cells (9; for review, see Vierstra [28]). Its amino acid sequence is one of the most conserved yet identified, being invariant in all animal species examined including humans, amphibians, insects, fish, and birds and differing by only three residues in yeast and higher plants (30 and references therein). Ubiquitin has at least three proposed functions that all stem from a novel posttranslational modification in which ubiquitin is enzymatically conjugated to other cellular proteins (6, 13). Conjugation requires ATP and occurs via an unusual peptide linkage between the terminal glycine carboxyl group on ubiquitin and α- and ϵ-groups on the various target proteins. In reticulocytes, the multienzyme system required for ubiquitin ligation has been identified and characterized (15).

In the cytoplasm, formation of ubiquitin conjugates appears to serve as a committed step for energy-dependent degradation of many intracellular proteins including those with short half-lives and/or structural abnormalities (6, 13). In this pathway, selective ubiquitination results in the rapid degradation of the modified protein with the concomitant release of free ubiquitin without digestion. This proposal has been substantiated by: (a) observations that ubiquitinated proteins are degraded much faster both *in vivo* and *in vitro* than their unmodified counterparts (14, 16, 17); (b) the demonstration that a temperature-sensitive mutant derived from a mouse carcinoma cell line is unable to degrade short-lived proteins at nonpermissive temperatures as a direct consequence of a thermolability in one of the enzymes requires for ubiquitin conjugation (3, 5); and (c) identification of proteolytic activities that specifically degrade ubiquitinated proteins (18).

In the nucleus, a role of ubiquitin in altering gene expression through selective modification of specific histones also appears evident. This role was first suggested from observations that the most abundant ubiquitin adducts in mammalian cells are modified forms of two specific histones, H2A and H2B (7). Variations in the steady state levels of these ubiquitinated histones as a function of cell cycle (22, 23) and transcriptional activity (8) and their selective occurrence within actively transcribed regions of chromatin (20) implicate this conjugation as a way to modify nucleosome structure and as a result facilitate gene expression.

A third possible role for ubiquitin conjugation has come from recent observations that the membrane-bound homing receptor on lymphocytes is actually a ubiquitin conjugate (27). Competition experiments with monoclonal antibodies to ubiquitin implicate the ubiquitin moiety as being directly involved in the surface recognition of this receptor. As a result, ubiquitin modification of plasma membrane-bound proteins also may be involved in cell-cell interactions.

Recently, we have begun to investigate the physiological roles of ubiquitin in higher plants. Initial characterizations demonstrated that ubiquitin isolated from oats (*Avena sativa*) is remarkably homologous to the animal form (29, 30). Further support was provided by x-ray crystallographic analyses which showed that oat, yeast, and human ubiquitin have near identical crystal structures (31). However, a prerequisite for ubiquitin to function in plants as it does in animals is the capacity to form ubiquitin-protein conjugates. Here, we demonstrate that plants have this ability with the detection *in vitro* of an ATP-dependent, enzymatic activity capable of ligating ubiquitin to other plant proteins.

MATERIALS AND METHODS

Reagents. Human and oat ubiquitin were purified according to the methods of Haas and Wilkinson (10) and Vierstra *et al.* (29), respectively. HPLC analyses (4) indicated that >90% of the ubiquitin in both the human and oat preparations was undegraded. Ubiquitin was radiolabeled with ^{125}I by the chloramine-T method as previously described (1). Initial specific radioactivity of the preparations of [^{125}I]ubiquitin was 3.9 to 4.5 × 10^3 cpm/pmol. Carrier-free [^{125}I]Na (5.6 × 10^8 Bq/μg) was a product of Amersham. Nucleotides, leupeptin, hexokinase, and phospho-

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creatine kinase were purchased from Sigma.

Plant Materials. Plant species used were oats (*Avena sativa* [*L.*] cv Garry), rye (*Secale cereale* [*L.*] cv Balboa), wheat (*Triticum aestivum* [*L.*] cv Scotty), corn (*Zea mays* [*L.*] cv hybrid MS WF9 × Bear 38), barley (*Hordeum vulgare* [*L.*] cv Morex), Peas (*Pisum sativum* [*L.*] cv Little Marvel), soybean (*Glycine max* [*L.*] *Merr.* cv Simpson), sunflower (*Helianthus annuus* [*L.*] cv Greystrip) and zucchini squash (*Cucurbita pepo* [*L.*] cv Black Beauty). Etiolated seedlings were grown at 24°C at near saturating humidity in darkness for 4 to 7 d depending on the species. Green oat seedlings were grown under continuous fluorescent light at 25°C for 9 to 10 d. After harvest, tissue was chilled to 4°C and homogenized with 50 mM Tris-HCl, 1 mM Na_4EDTA, and 14 mM 2-mercaptoethanol (pH 8.0) 4°C. For experiments involving *N*-ethylmaleimide or 2-mercaptoethanol, 10 mM sodium metabisulfite was used instead of 2-mercaptoethanol in the homogenization buffer. Tissue to buffer ratios (g/ml) were 1:2 for etiolated tissue, 1:3 for green leaves, and 1:4 for dry seeds. Seeds were ground into a fine powder with a mortar and pestle at 77 K prior to homogenization. Extracts were centrifuged for 5 min at 50,000*g* and the supernatants were filtered through cheesecloth and centrifuged again for 5 min at 50,000*g*. This crude soluble extract was made 200 μM leupeptin and used for all subsequent experiments. For experiments involving an unclarified crude extract, the initial homogenate was used immediately after filtering through cheesecloth.

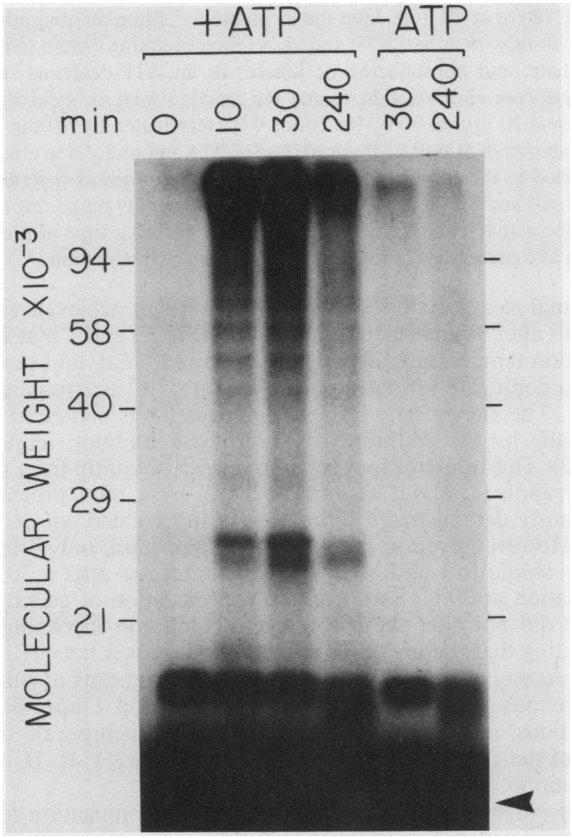

FIG. 1. Detection of ATP-dependent, ubiquitin conjugation in a crude extract of etiolated oats. ^{125}I-human ubiquitin was added to a crude oat extract along with either ATP and an ATP-regenerating system (creatine phosphate and phosphocreatine kinase [+ATP]) or an ATP-depleting system (deoxyglucose and hexokinase [−ATP]). The reaction mixtures were incubated at 30°C and at various times the reactions were terminated by boiling in an equal aliquot of SDS-PAGE sample buffer. The samples were then subjected to SDS-PAGE and autoradiography. Arrow to the right indicates the position of free ubiquitin.

Table I. *Effect of Nucleotides and Inhibitors on Ubiquitin Conjugation in Vitro*

Additions	Ubiquitin-Conjugating Activity
	% ± SD[a]
Experiment I	
Mg^{2+}ATP + creatine phosphate + kinase	100
Mg^{2+}ATP	78 ± 7
ATP	67 ± 1
Mg^{2+}	48 ± 5
Buffer control	47 ± 11
Mg^{2+}ADP	62 ± 6
Mg^{2+}CTP	32 ± 4
Mg^{2+}GTP	48 ± 4
Mg^{2+}TTP	55 ± 6
Mg^{2+}UTP	54 ± 6
Experiment II	
Mg^{2+}ATP + creatine phosphate + kinase	100
+ *N*-ethylmaleimide (5 mM)	27 ± 10
+ 2-mercaptoethanol (14 mM)	73 ± 10
+ 2-mercaptoethanol (140 mM)	37± 5

[a] Results for each treatment represent the average of three independent experiments and are expressed as a percentage of the ubiquitin conjugates formed in the presence of Mg^{2+}ATP, creatine phosphate, and phosphocreatine kinase. Nucleotide and Mg^{2+} concentrations were 0.2 and 0.5 mM, respectively.

Assay for Ubiquitin Conjugation. Unless otherwise stated, conjugation assays consisted of 40 μl of crude extract; 5 μl of ^{125}I-human ubiquitin (0.8 μg); and either 5 μl of 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 10 mM creatine phosphate, 50 mM Tris-HCl (pH 7.6, 20°C), and 1 μl (1 unit/μl) of phosphocreatine kinase for the ATP-stimulated activity or 5 μl of 5 mM $MgCl_2$, 1 mM DTT, 10 mM deoxyglucose, 50 mM Tris-HCl (pH 7.6, 20°C), and 1 μl (1 unit/μl) of hexokinase for the ATP-independent activity (24). The reaction mixtures were incubated at 30°C for various times and the reactions terminated by boiling in 50 μl of SDS-PAGE sample buffer containing 125 mM Tris-HCl, 10% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol, and 0.002% (w/v) bromophenol blue (pH 6.8, 25°C). Samples were subjected to discontinuous SDS-PAGE (19) on 13.5% acrylamide running gels (acrylamide:bisacrylamide ratio of 30:1). The gels were stained with Coomassie blue R, dried between two sheets of cellophane, and used for autoradiography. Quantitation of ubiquitin conjugates formed during each reaction was accomplished by liquid scintillation counting slices of the individual gel lanes after removing the lower portion of the gel containing free ubiquitin (determined from the autoradiograms).

RESULTS

Energy-Dependent Formation of Ubiquitin Conjugates. Ciechanover *et al.* (1) first demonstrated in reticulocyte lysates, an ATP-dependent, soluble activity that covalently ligates [^{125}I] ubiquitin to endogenous or exogenous proteins. Upon addition of ATP and an ATP-regenerating system (creatine phosphate and phosphocreatine kinase) or an ATP-depleting system (deoxyglucose and hexokinase) as described previously (24), we have detected a similar conjugating activity in crude extracts from etiolated seedlings of the higher plant, oats. This activity catalysed the energy-dependent ligation of ^{125}I-human ubiquitin to a variety of endogenous oat proteins (Fig. 1). (The ^{125}I-labeled contaminant above free ubiquitin present in all gel lanes [Figs. 1, 4] represents a modidied form of ubiquitin commonly found in purified preparations of human ubiquitin). Similar to that observed in reticulocytes, many conjugates formed in this manner

had molecular masses exceeding 100 kDa indicating that they represent multiply conjugated proteins. The stability of these conjugates to prolonged boiling (5 min) in SDS and 2-mercaptoethanol indicated that the linkage was covalent. The conjugating activity has a soluble distribution based on the observation that all the activity of the crude extract remained in the supernatant after centrifugation at 50,000*g*. Comparable results were also observed when ^{125}I-oat ubiquitin was used as the substrate (R. D. Vierstra, unpublished data). However, because our oat ubiquitin preparations contained several minor contaminants (not observable after Coomassie staining) that were effectively labeled with ^{125}I, human ubiquitin was used for further studies.

Characterization of the Ubiquitin Conjugation System. Because the addition of ATP and an ATP-generating system increased the amount of ubiquitin in conjugates 4- to 8-fold relative to the amount obtained with the ATP-depleting system (Fig. 1), we concluded that conjugation is an energy-requiring process. Comparison of various nucleotides indicated that the reaction is more specific for Mg^{2+}ATP (Table I). Conjugation was stimulated by relatively low concentrations of ATP with 0.2 mM required for optimal activity. Other nucleotides tested, CTP, GTP, TTP, AND UTP showed little or no stimulation of conjugate formation above the control level. Substantial ubiquitin conjugation occurred even in the absence of added energy (Buffer Control, Table I); about half the activity observed with the addition of ATP and the ATP-regenerating system. From the data of Quail (25), we calculate that the ATP concentration of such oat extracts would be approximately 50 μM immediately after homogenization but should decline rapidly from this value as the result of endogenous phosphatase activity. Therefore, even at the low ATP concentrations expected to be present in such crude homogenates at the time of assay, significant ubiquitin conjugation could still occur.

Kinetic analyses of ubiquitin ligation at various temperatures revealed that the reaction was strongly temperature dependent.

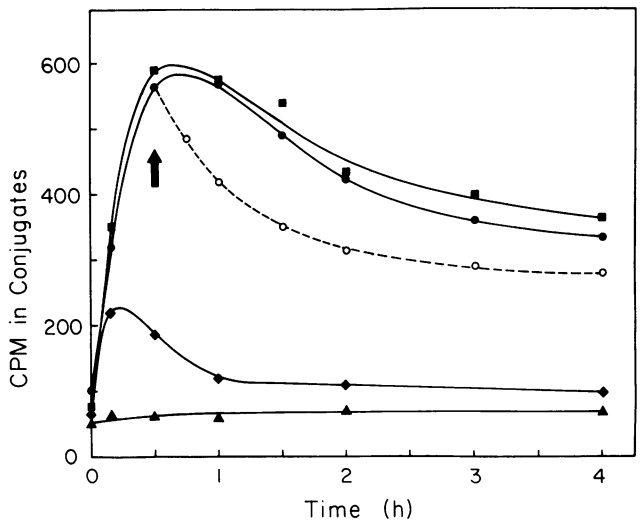

FIG. 2. Kinetics of ubiquitin conjugation in a crude extract of etiolated oats. A crude oat extract was prepared with (▲, ●, ■) or without 200 μM leupeptin (◆) and incubated with ^{125}I-human ubiquitin and either: (■, ◆) ATP and an ATP-regenerating system (creatine phosphate and phosphocreatine kinase); (●) ATP alone; or (▲) an ATP-depleting system (deoxyglucose and hexokinase). At the position of the arrow, deoxyglucose and hexokinase were added to an aliquot of the reaction containing ATP alone and incubated further (○). The reaction mixtures were incubated at 30°C and at various times the reactions were terminated by boiling in an equal aliquot of SDS-PAGE sample buffer. The amount of free ubiquitin converted to the conjugated form was then determined as described in "Materials and Methods."

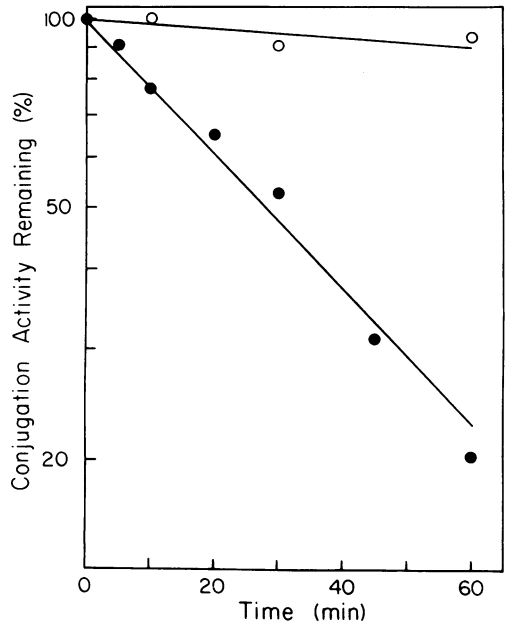

FIG. 3. Inactivation of the ATP-dependent, ubiquitin-conjugating activity in a crude extract of etiolated oats during incubation. A crude oat extract was made 200 μM in leupeptin and incubated for various times at 30°C (●) or at 4°C (○). After the incubation, ^{125}I-human ubiquitin was added along with either; ATP and an ATP-regenerating system (creatine phosphate, and phosphocreatine kinase) or an ATP-depleting system (deoxyglucose and hexokinase) and the reaction mixture incubated an additional 30 min at 30°C. Reactions were terminated by boiling in an equal aliquot of SDS-PAGE sample buffer. The amount of free ubiquitin converted to the conjugated form was then determined as described in "Materials and Methods." Percent conjugating activity remaining represents the activity which is stimulated by ATP for each time of preincubation as a percentage of that obtained without preincubation.

Maximal steady state levels of conjugates was achieved within 30 min after ubiquitin addition at 30°C (Fig. 2) and 2 h at 21°C. Reaction temperatures above 30°C were inhibitory and resulted in less conjugate formation (40% less at 37°C as compared to 30°C). The steady state level of conjugates was enhanced substantially by the addition of the protease-inhibitor, leupeptin (Fig. 2). This inhibitor may serve to protect ubiquitin from rapid inactivation ($t_{½}$ about 10 min at 27°C) by a thiol protease(s) previously demonstrated to be present in etiolated oat extracts (28). However, even in the presence of leupeptin, only 4 to 5% of the ubiquitin could be trapped in conjugates after a 30 min incubation at 30°C. Reducing the concentration of added ubiquitin did not alter the percentage of ubiquitin in conjugates, indicating that the low level of incorporation was not the result of saturating levels of ubiquitin or limiting amounts of suitable endogenous substrate proteins for ubiquitination. Under similar conditions, reticulocyte lysates were able to incorporate 60 to 80% of the exogenous ubiquitin into conjugates (1; R. D. Vierstra, unpublished data).

One potential reason for the low level of conjugation found with etiolated oat extracts is that the conjugating activity is extremely labile. Preincubation of the crude extract prior to ubiquitin and ATP addition resulted in a rapid, first order decline in the extract's ability to perform ATP-dependent conjugation (Fig. 3). The rate of inactivation was strongly temperature dependent with a $t_{½}$ of 30 min at 30°C. This inactivation was not altered by preincubation with either 200 μM leupeptin or 2 mM phenylmethylsulfonyl fluoride which would help preclude proteolysis by thiol- or serine-dependent proteases as the mechanism of inactivation.

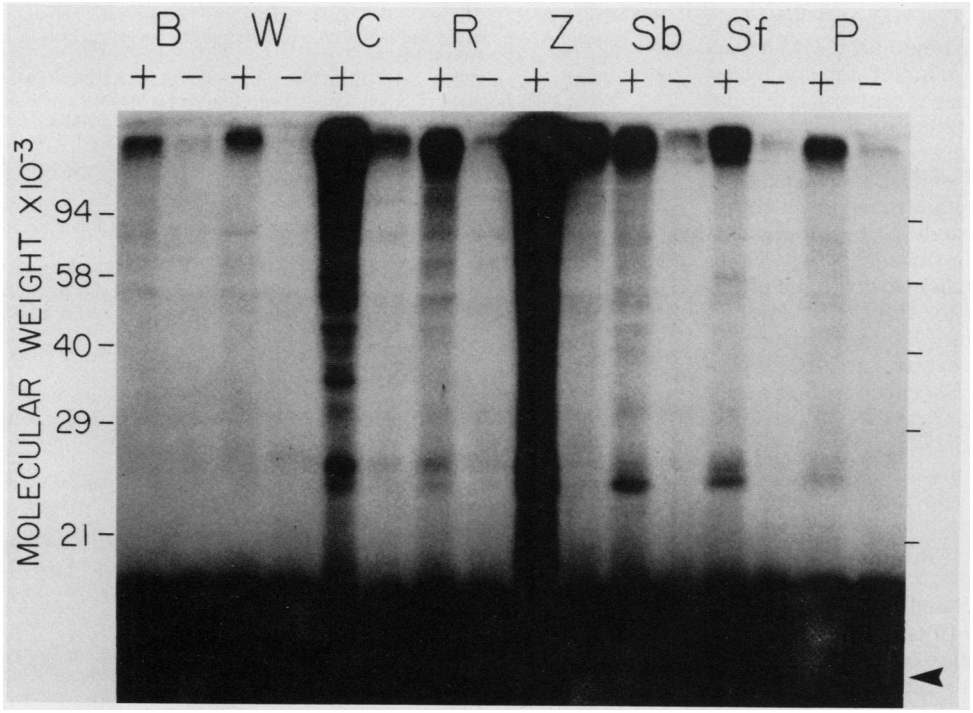

FIG. 4. Detection of ATP-dependent, ubiquitin conjugation in crude extracts of various plant species. Crude extracts were prepared from etiolated seedling of barley (B), wheat (W), corn (C), rye (R), zucchini squash (Z), soybean (Sb), sunflower (Sf), and pea (P). ^{125}I-human ubiquitin was added to the extracts along with either: ATP and ATP-regenerating system (creatine phosphate and phosphocreatine kinase [+]) or an ATP-depleting system (deoxyglucose and hexokinase [−]). The reaction mixtures were incubated for 30 min at 30°C and the reactions were terminated by boiling in an equal aliquot of SDS-PAGE sample buffer. The samples were then subjected to SDS-PAGE and autoradiography. Arrow to the right indicates the position of free ubiquitin.

Further kinetic analyses indicated that after incubations longer than 30 min at 30°C, the steady state level of conjugates declined (Fig. 2). The profile of conjugates on SDS-PAGE did not change during this loss indicating that all conjugates were degraded at the same rate. Because depletion of the ATP pool after conjugate formation by the addition of deoxyglucose and hexokinase to reaction mixtures containing ATP failed to prevent this decline (Fig. 2), it suggests that the degradation (or disassembly) process does not require high ATP concentrations. The loss of conjugates was also unaffected by the protease inhibitors, 2 mM phenylmethylsulfonyl fluoride and 200 μM leupeptin.

In reticulocytes, the mechanism of ubiquitin ligation involves first adenylation of the carboxy-terminus of ubiquitin and then transfer of activated ubiquitin to a thiol site on the ubiquitin-activating enzyme, E1 and subsequently to thiol sites on a class of ubiquitin carrier proteins, E2s (2). As a result, sulfhydryl-reactive agents and thiols are potent inhibitors of ubiquitin conjugation in such lysates by modification of or substituting for the thiol sites on E1 and E2s. We find that such agents also inhibit conjugate formation in oat extracts. Preincubation of extracts with either 5 mM *N*-ethylmaleimide or 140 mM 2-mercaptoethanol effectively impaired the ATP-dependent conjugating activity (Table I).

Detection of ATP-Dependent Ubiquitin Conjugating Activities in Various Tissues and Plant Species. In addition to observing ubiquitin conjugation in etiolated oat extracts, we have also detected this activity in extracts from green oat leaves and dry oat seeds (R. D. Vierstra, unpublished data). The activities in both tissue extracts are weak, about 10-fold lower than that observed in etiolated extracts when expressed on a per mg protein basis. We have also surveyed several other plant species for ATP-dependent ubiquitin ligation and found that etiolated tissue from all species tested have such an *in vitro* activity (Fig. 4). Plant species tested include barley, wheat, corn, rye, zucchini squash, soybean, sunflower, and pea. The extent of conjugation varies substantially from species to species with zucchini squash being the most active and barley the least active.

DISCUSSION

In this study, we demonstrate that higher plants contain an ATP-dependent activity capable of forming ubiquitin conjugates with endogenous proteins *in vitro*. These data support the previous immunological evidence suggesting that higher plants contain ubiquitin conjugates *in vivo* and as a result have enzymic activities capable of ubiquitin ligation (29). Therefore, higher plants can perform the necessary steps required for ubiquitin to function *in vivo*, including its involvement in protein turnover and gene regulation (6, 13). The ability to substitute human ubiquitin for the oat protein in these assays was expected from previous enzymic and structural studies indicating the the two proteins are nearly identical (29–31). Using the incorporation of [^{125}I]ubiquitin into higher mol wt proteins and subsequent separation on SDS-PAGE as an assay, it should be possible to isolate and characterize the enzymic activities in plants responsible for conjugation.

The ligation system described here is analogous to the well characterized reticulocyte lysate system (1, 2, 15, 16), including its soluble distribution, specificity for Mg^{2+}ATP, and inhibition by sulfhydryl-reactive agents. However, the activity in etiolated plant extracts is much lower than that observed in reticulocytes as a result of several, possibly cumulative effects. First, previous studies with reticulocyte lysates used a partially purified extract in which endogenous ubiquitin pool was depleted (fraction II [1]) which may have enhanced the use of exogenous ubiquitin. Second, preincubation studies indicate that the plant activity is extremely labile resulting in a substantial loss of activity during the assay period. The nature of this lability is unclear but two possibilities are inactivation by proteolysis or by polyphenol modification. Third, as previously demonstrated (29), plant extracts contain proteases which rapidly inactivate the ubiquitin substrate. It is apparent that the addition of leupeptin helps alleviate this problem, but whether there is sufficient residual activity to impair conjugation is unknown. It should be noted that a similar proteolytic artifact led to the previous inability to detect ubiquitin conjugating activities in liver extracts (11). Fourth, oat extacts contain activities which degrade conjugates once formed, thus lowering the measured steady state level. Such degradation could proceed by either a nonspecific protease, an ATP-dependent, conjugate-specific protease similar to that characterized in reticulocytes by Hough *et al.* (18), or an isopeptidase

homologous to that found in reticulocytes which cleaves only the isopeptide bond and regenerates free ubiquitin (12). The fact that high ATP concentrations are not required for conjugates breakdown would suggest that an ATP-dependent protease is not primarily involved. Fifth, since human ubiquitin is not identical to the oat form, the subtle structural differences in the human protein could alter the kinetic efficiency of the plant conjugating enzymes. Sixth, plants may actually contain much lower conjugating activities. If the conjugating system in higher plants is restricted mainly to the cytoplasm, then this low activity could be partially explained by the proportionately low volume of this cell compartment (20–30%) relative to the total cell volume of an etiolated plant cell. The many ways conjugation can be affected by components in crude plant extracts underscores the complexity of the system and problems associated with its future dissection.

The relevance of ubiquitin conjugation to the physiology and development of higher plants is as yet unresolved. The striking homology of plant ubiquitin (29–31) and related conjugation reactions (this report) to that characterized in mammalian cells and their ubiquitous presence in all plant species examined makes it highly likely that this modification serves similar functions in plants as it does in animals. Based on the number of diverse conjugates formed both *in vitro* (this report) and *in vivo* (29), it is reasonable to assume that ligation is not restricted only to a few specific plant proteins. With regard to ubiquitin's functions, we have recently discovered that the photomorphogenic photoreceptor, phytochrome, is rapidly ubiquitinated *in vivo* after phototransformation to the far-red light absorbing form (26). Since phytochrome is rapidly degraded after this photoconversion,it suggests that this plant protein is degraded via ubiquitinated intermediate(s).

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