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Conjugation of Ubiquitin to Proteins from Green Plant Tissues[1]

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ABSTRACT

Conjugation of the polypeptide ubiquitin to endogenous proteins was studied in oat (*Avena sativa* L.) plants, and particularly in green tissues. Conjugating activity in leaf extracts was different from that in root extracts, and in both was less than in etiolated tissue. The conjugates were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and their formation was both time- and ATP-dependent and had a pH optimum of about 8.2. The assay had a high affinity for ATP with a probable K_m of less than 50 micromolar. The ubiquitin conjugating system was also shown to be present in isolated chloroplasts, and ubiquitin could be conjugated to endogenous proteins of lyzed chloroplasts in which the ATP concentrations were reduced by preincubation or desalting. SDS-PAGE analysis led to the suggestion that the large and small subunits of ribulose-1,5-bisphosphate carboxylase (RuBPCase) may be able to be ubiquitinated, and we have shown that ubiquitin can stimulate the *in vitro* breakdown of ^{125}I-labeled RuBPCase. These results invite the speculation that ubiquitin may be involved in the regulation of protein turnover in green plants.

The major proteolytic enzymes that have in the past been associated with protein turnover and breakdown in plant tissues are the acid proteases in the plant cell vacuole (2, 26). However, increasing evidence indicates that in both animals and plants, ATP-dependent proteases elsewhere in the cell may be important in controlling protein turnover during steady-state conditions (7, 9, 12, 27).

In animal cells and microorganisms, some ATP-dependent proteases specifically degrade proteins which have been targeted by the small polypeptide ubiquitin (3, 6, 13, 14). Ubiquitin is conjugated to the target protein, also in an ATP-dependent reaction, by a complex consisting of three enzymes (6). The consequent ubiquitin-protein complex is subject to proteolysis, ubiquitin being subsequently released. This ubiquitin-dependent pathway for protein breakdown has been suggested as one important means of controlling protein turnover in the cell (11) and might be involved in the heat shock response. Many features of this pathway have been shown to exist in etiolated plants and germinating seeds (27–29). Although there has been some discussion of ubiquitin in green plants (27), including data from the green alga *Chlamydomonas* (23), the potential role of ubiquitin in regulating protein turnover in green tissues has not been explored, with most work having been done on etiolated tissues. One role of ubiquitin in plants may be regulation of phytochrome turnover (15, 22).

In the green plant the most abundent protein is RuBPCase,[3] located in the chloroplasts. Catabolism of RuBPCase begins soon after leaf expansion ceases and accelerates during senescence (4). Although chloroplast proteins are degraded *in situ*, the specific proteases responsible for the turnover of chloroplastic proteins during senescence are just beginning to be identified (5, 19, 20, 24). The presence of ubiquitin in most plant tissues tested (27) invites the speculation that it may be involved in regulation of protein turnover in green tissues, and particularly of chloroplastic proteins. Whereas little is known about ATP-dependent proteases in plants, in chloroplasts, newly synthesized proteins have been shown to be degraded by unspecified ATP-dependent proteases (17–19).

The work presented here was undertaken to investigate further the operation of the ubiquitin system in oat plants. Ubiquitin in oats has been previously identified and sequenced (30). Our particular aim has been to study conjugation of ubiquitin to proteins in light grown plants.

MATERIAL AND METHODS

Plant Material

Oats (*Avena sativa* L. cv Makuru) were germinated in a general seedling soil mixture (Western Nurseries Ltd, Auckland, N.Z.), and grown either in darkness for 7 d or in the light for 10 d. The temperature was 23 ± 2°C, and the irradiance at plant level 300 μmol m^{-2} s^{-1}, the light source being a 400 W M400/C/BU-HOR lamp (Sylvania-Canada). A 16 h d was maintained.

Plant Extraction

For a crude extract, plant material was homogenized in a glass grinder in ice-cold Tris/Mes/KOH buffer (0.1 M [pH 7.2], tissue weight to volume 1:4). The homogenate was filtered through two layers of cheesecloth and centrifuged at

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[3] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; APMSF, (4-amidinophenyl)-methanesulfonyl fluoride.

25,000*g* for 10 min. The supernatant was used directly for ubiquitin binding assays. Protein was measured by the method of Bradford (1).

Chloroplast Isolation

Approximately 20 g of green leaves were cut into 2 mm segments and homogenized in 20 mL of ice-cold grinding media (0.35 M sucrose, 0.05 M Hepes, 0.01 M $KHCO_3$, 0.002 M NaEDTA, 0.01 M KCl, 0.005 M $Na_4P_2O_7$, 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, 0.004 M DTT, and 0.0002 M ADP, pH adjusted to 7.5 with KOH) in a Virtis homogenizer for 2 s at 45,000 rpm. The homogenate was filtered through six layers of cheesecloth and one layer of Mira cloth. After centrifugation at 2,500*g* for 5 min (Sorvall RC-2B centrifuge, HB-4 rotor), the supernatant was discharged and the pellet resuspended in 2 mL grinding medium. The chloroplast suspension was placed on top of a Percoll gradient in a 12 mL centrifugation tube (2 mL 60%, overlayed by 6 mL 40% Percoll, all in grinding medium). After centrifugation at 12,000*g* for 1 min the lower band was removed and resuspended in 20 mL grinding medium, and the chloroplasts were pelleted by centrifugation at 2,500*g* for 5 min. For ubiquitin binding assays the chloroplast pellet was lysed in 1 mL Hepes/KOH buffer (0.01 M [pH 7.2]) and centrifuged at 12,000*g* for 2 min, whereafter the supernatant was used for the binding assay.

Radiolabeling

Ubiquitin or RuBPCase was labeled with ^{125}I by the chloramine T method as described by Ciechanover *et al.* (3), except that the reaction was terminated by the addition of 10 μL cysteine (0.8 mg mL^{-1} in 0.05 M phosphate buffer [pH 7.5]). After purification on a Sephadex G-25 column, the specific activity was between 0.8 and 3.5×10^6 cpm μg^{-1} of ubiquitin and 28×10^6 cpm μg^{-1} of RuBPCase.

Carrier-free [^{125}I]Na (509 MBq μg^{-1}) was obtained from Amersham, UK. Ubiquitin (bovine) and RuBPCase (spinach) were obtained from Sigma.

Ubiquitin Binding Assay

The conjugation assay consisted of 40 μL extract, 10 μL Tris/Mes/KOH (0.25 M [pH 8.2]), and either 3 μL of 50 mM ATP and 5 μL of 0.005 M $MgCl_2$, 0.001 M DTT, 0.01 M creatine phosphate, 0.05 M Hepes/KOH (pH 8.2), and 2 μL (1 unit mL^{-1}) creatine phosphokinase (Sigma) for ATP-stimulated activity, or 5 μL of 0.005 M $MgCl_2$, 0.001 M DTT, 0.01 M deoxyglucose, 0.05 M Hepes/KOH (pH 8.2), and 5 μL (0.5 unit mL^{-1}) hexokinase (Sigma) for ATP-independent activity. The assay was performed at 30°C for various times. At the termination of the reaction, most of the unbound ubiquitin was separated from the reaction mixture by placing 50 μL on a small Sephadex G-100 column (1.6 mL of Sephadex G-100 in a 1 mL micropipette tip) and eluted with Hepes/KOH buffer (0.01 M [pH 7.2]). Fraction 400 to 800 μL was collected and a 50 μL sample was used to determine cpm, and the rest of the sample freeze-dried for later identification of labelled conjugates by SDS-PAGE.

In experiments with lyzed chloroplasts, where we wished to deplete the extract further of ATP, 1 mL of the chloroplasts was placed on a Sephadex G-25 column (1 × 7 cm). The proteins were eluted with Hepes/KOH buffer (0.01 M [pH 7.2]), with the first 1.2 mL of the protein fractions being collected. This fraction was used in ATP-depleted ubiquitin binding assays. Depletion of ATP was confirmed with ^{32}P-ATP.

Ubiquitin-Dependent Proteolysis of RuBPCase

The binding assay was similar to that above except for the use of 10^8 cpm ^{125}I RuBPCase and 5 μg unlabeled ubiquitin in the ubiquitin-dependent assay. The assay was terminated by adding TCA (5% final concentration), and after 1 h on ice, the samples were centrifuged at 12,000*g* for 5 min, whereafter aliquots of the supernatant were assayed for ^{125}I by liquid scintillation spectrophotometry.

Electrophoresis and Autoradiography

Samples were prepared for electrophoresis by solubilization in equal volumes of SDS buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 10% mercaptoethanol [pH 6.8]), followed by boiling for 5 min. Polypeptides and ubiquitin-polypeptide conjugates were separated on 10, 13, or 15% SDS-polyacrylamide gels (16). High and low mol wt markers (Bio-Rad) were run with each gel. Gels were stained either with Coomassie blue or by silver staining. Detection of ^{125}I labeling was by autoradiography using x-ray film (Agfa-Gevaert) in cassettes with enhancing screens, held at −80°C.

RESULTS

When ^{125}I ubiquitin was applied to homogenates from etiolated or light-grown oat plants, many of the proteins became labeled, and it was possible to separate these proteins by SDS-PAGE. Although the mol wt of protein/ubiquitin conjugates can be estimated, it is not possible to determine the mol wt of the original protein itself, since migration on the gel depends on the site of ubiquitin attachment. Conjugates are shown as labeled bands on the gels at M_r above that of ubiquitin itself (8,600).

Conjugation of ^{125}I ubiquitin to proteins in purified leaf homogenates from green oat plants was both ATP- and time-dependent (Fig. 1). The major difference between binding in extracts from green and etiolated tissues was in the more substantial conjugation to high mol wt proteins from etiolated leaves (Fig. 1). This was also the case in other nonchlorophyllous tissue such as roots. Roots from hydroponically grown green plants were excised, and the terminal 1 cm portion homogenized and used for the ubiquitin binding assay. Whereas the pattern of conjugation in roots was strong in the high mol wt region as seen in etiolated tissue, there was also binding in many low mol wt bands as seen in green leaves (Fig. 2). Nongreen tissue characteristically showed a much stronger ATP dependence than did green tissue.

A pH of 7.6 is commonly used for the ubiquitin binding assay in reticulocytes, and an optimum for extracts from wheat germ was approximately pH 8 (10). When the binding assay was performed on crude homogenates from green tissue,

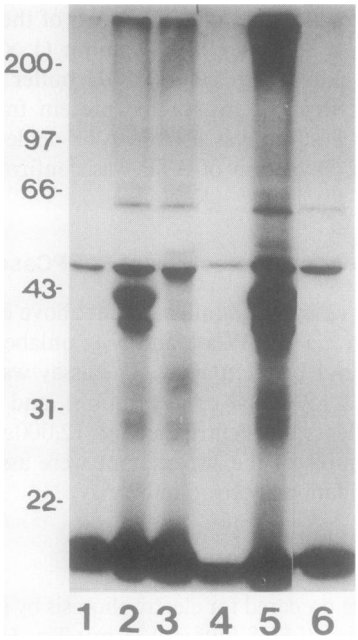

Figure 1. ATP- and time-dependent conjugation of ^{125}I-ubiquitin to proteins in a crude extract from green (lanes 1–3) or etiolated (lanes 4–6) oat leaves. The assay was stopped at 0 (lanes 1, 4) or 60 (lanes 2, 3, 5, 6) min, and was carried out in the presence (lanes 1, 2, 4, 5) or absence (lanes 3, 6) of ATP (see "Materials and Methods"). The assay was performed with 60 or 80 μg protein for green or etiolated tissue, respectively, and 40% thereof was loaded on the gels. The heavily labeled band at the base of the gel is unbound ubiquitin. (M_r (×1000) are shown on the left.)

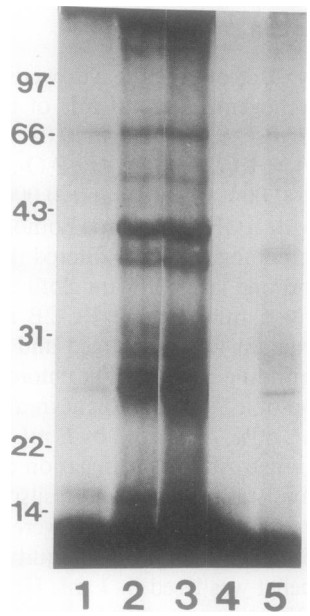

Figure 2. ATP- and time-dependent conjugation of ^{125}I-ubiquitin to proteins in a crude extract from oat roots. The assay was stopped at 0 (lanes 1, 4), 30 (lane 2), or 90 (lanes 3, 5) min, and was carried out in the presence (lanes 1–3) or absence (lanes 4, 5) of ATP (see "Materials and Methods"). The assay was performed with 45 μg protein and 40% thereof was loaded on each lane. The heavily labeled band at the base of the gel is unbound ^{125}I-ubiquitin. (M_r (×1000) are shown on the left.)

in the pH range of 5.5 to 9.5, optimum binding occurred between pH 8.0 and 8.5 (Fig. 3), with more than 80% optimal binding occurring between pH 7.6 and 8.6.

These results suggest that there is substantial conjugation of ubiquitin to proteins in green leaf tissue. Since up to 50% of leaf protein is RuBPCase, we investigated whether or not the ubiquitin binding system was present and able to cause degradation of RuBPCase in isolated oat chloroplasts. By using [^{125}I]RuBPCase it was possible to show a small but significant ubiquitin-dependent increase in degradation of this protein when an homogenate from green oat leaves was used as a source of the conjugation enzymes (Fig. 4). There was an increase in soluble ^{125}I in the assay over 90 min.

The above results led us to investigate further the capacity of chloroplasts to perform ubiquitin-dependent breakdown. Chloroplasts were isolated from green oat leaves, lysed, and used as substrate in the assays. Binding of ^{125}I-ubiquitin to chloroplast proteins increased over an assay time of 12 h, but it was not found to be ATP-dependent. Because the chloroplasts were isolated from green tissue under conditions that did not deplete them of ATP, we tested whether preincubation of lysed chloroplasts in ATP-depleting conditions (hexokinase and deoxyglucose) would affect the amount of conjugation. An incubation period of up to 30 min caused a substantial increase in ATP-dependent binding of ^{125}I-ubiquitin to chloroplast proteins (Fig. 5). A further increase in preincubation time reduced conjugation. However, although preincubation

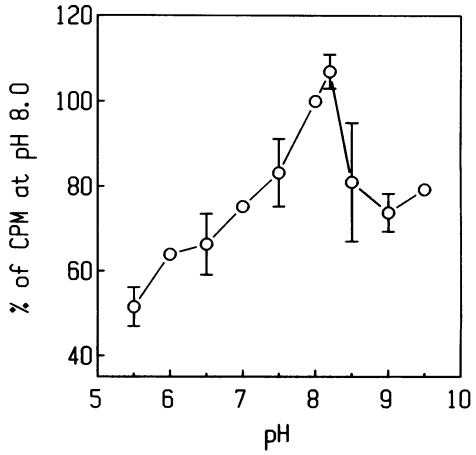

Figure 3. Effect of pH on ATP-dependent conjugation of ^{125}I-ubiquitin to proteins in extracts from green oat leaves. Each assay contained 40 to 80 μg protein, and was run for 2 h. Data are the means from three experiments, bars represent ±SE.

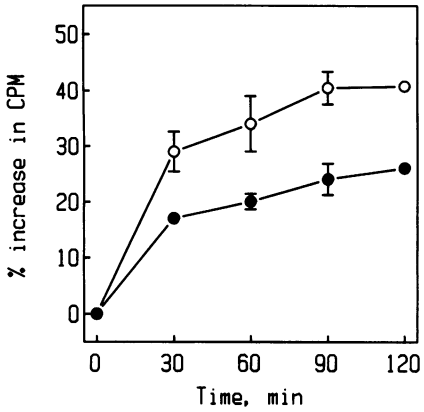

Figure 4. Ubiquitin-dependent breakdown of ^{125}I-RuBPCase. Lysed chloroplasts (40–80 μg protein) were used as a source of binding enzymes. Five μg ^{125}I-RuBPCase was added in the presence (○) or absence (●) of 10 μg ubiquitin. The reaction was stopped with TCA at the indicated times, and the radioactivity in the supernatant determined (see "Materials and Methods"). Initial cpm = 132800 ± 5560. Data are the means of four experiments, bars represent ±SE.

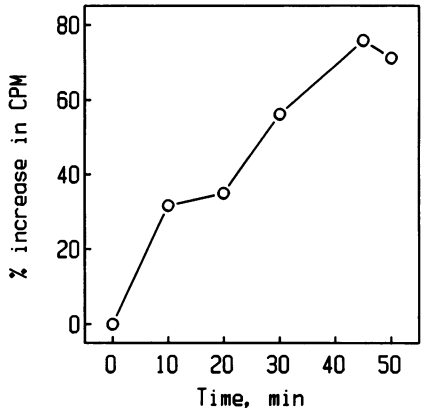

Figure 6. Time-dependent conjugation of ^{125}I-ubiquitin to proteins from lysed chloroplasts. The reaction mixture was preincubated for 30 min before the addition of ATP and labeled ubiquitin. It was then run for the given times; 120 μg protein was used in each assay. Initial cpm = 3382. The data are from one of three experiments.

almost always increased the levels of ATP-dependent conjugation, the time before subsequent deactivation occurred varied considerably between replicate experiments (data not shown). When a 30 min preincubation period was used, the optimal binding assay time was about 45 min (Fig. 6). Although preincubation caused the ubiquitin binding to become ATP- and time-dependent, it caused a loss of the high mol wt conjugates (Fig. 7). Desalting of the lysed chloroplasts on a Sephadex G-25 column also made the assay ATP-dependent, but without loss of high mol wt conjugates. The capacity for conjugation of ubiquitin to proteins was found not only in oat leaf chloroplasts, but also in spinach, pea, and amaranthus leaf chloroplasts (data not shown).

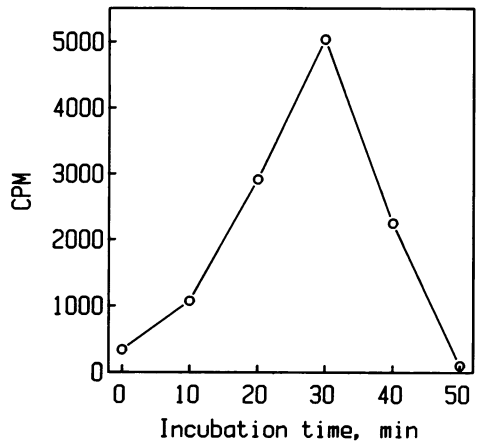

Figure 5. Effect of preincubation on ATP-dependent conjugation of ^{125}I-ubiquitin to proteins from lyzed chloroplasts. The reaction mixture was preincubated under ATP-depleting conditions for the given times at 30°C, whereafter 500 μM ATP and the labeled ubiquitin were added. The conjugating assay was then run for 45 min; 120 μg protein was used in each assay. The data are from one of three experiments.

DISCUSSION

The ubiquitin-binding assay used in the present work is an assay of the activity of the three-enzyme complex required for conjugation of ubiquitin to target proteins. It is clear that these enzymes are present in green leaf tissue (Fig. 1), in root tips (Fig. 2), and in chloroplasts in particular (Fig. 7). Previous work by Vierstra and colleagues (10, 27–29) concentrated on etiolated seedlings, or model systems such as wheat germ. Vierstra (27) found that conjugating activity in green oat tissue was less than in etiolated tissue and much lower than that found in animals such as in the reticulocyte lysate system, although no data were given. It was possible to show and measure substantial conjugating activity in green tissues, by removing a large proportion of residual, unbound ^{125}I-ubiquitin at the end of the assay by gel filtration. This allowed better visualization of conjugates by SDS-PAGE; otherwise, the high levels of unbound ^{125}I-ubiquitin can obscure autoradiographic development of conjugates. A complete separation of free ubiquitin and conjugates was not possible by gel filtration. Our data show that less ^{125}I-ubiquitin was bound to high mol wt proteins in green than in etiolated homogenates, two major bands having mol wt of 38,000 and 41,000 (Fig. 1). The major differences between root and green tissue extracts were in the greater conjugation of high mol wt proteins in the root tips, and in the ATP-dependence (Fig. 2). Vierstra (27) has pointed out that even in etiolated tissues, some 50% of the total conjugating activity occurred in the absence of added ATP or ATP-generating system. The high endogenous levels of ATP meant that there were difficulties in recording ATP dose responses in green extracts. Some preliminary data from etiolated extracts indicated a possible K_m of less than 50 μM (35 μM, data not shown). This is close to the suggested concentration of ATP (50 μM) likely to be present in etiolated extracts (27), and similar to the K_m reported for other systems such as reticulocytes (3). It is obvious that if the K_m is so low,

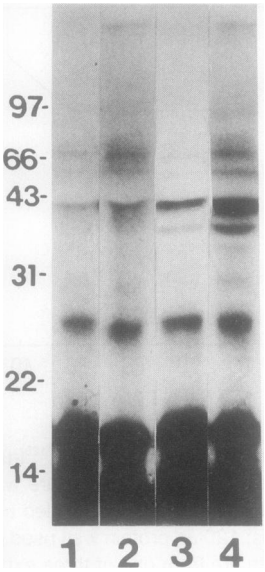

Figure 7. ATP- and time-dependent conjugation of ^{125}I-ubiquitin to proteins from lysed chloroplasts. The reaction mixture was preincubated for 30 min before the addition of ATP and labeled ubiquitin. The assay was carried out in the absence (lanes 1, 2) or presence (lanes 3, 4) of 500 μM ATP, and run for 0 (lanes 1, 3) or 60 (lanes 2, 4) min. Each assay contained 120 μg protein, and 40% thereof was loaded on each lane. (M_r (×1000) are shown on the left.)

it will result in difficulties in depleting ATP concentrations sufficiently to show ATP-dependence in green oat leaves where the ATP is at least 100 μM (25).

The pH optimum for the presence of conjugates was about 8.2 (Fig. 3) which is different from that in the mammalian system (about 7.6). Although considerable conjugation occurs at lower pH values, these high pH values are found only in the stroma in the chloroplasts and the mitochondria matrix. Ubiquitin-dependent proteolysis has been described in reticulocyte mitochondria (21), and our results show not only that RuBPCase can undergo ubiquitin-dependent degradation *in vitro* (Fig. 4), but also the existence of the conjugating enzymes in chloroplasts (Figs. 5, 6, and 7).

Because of the difficulties in showing ATP dependence for ubiquitin binding in isolated chloroplasts, lysed chloroplasts were preincubated with the ATP-generating or -depleting enzyme system for up to 50 min. It was found that preincubation for up to 30 min increased the amount of ^{125}I-ubiquitin bound to proteins (Fig. 5). However, the optimum time period for preincubation varied considerably between experiments, precluding this method from routine use. Contrary to our results, an active carboxylase has been described in homogenates from etiolated tissue (27). After a preincubation of 30 min, a time-dependent incorporation of ^{125}I-ubiquitin into proteins continued for at least the next 45 min (Fig. 6). Gel filtration was also used in another attempt to decrease the endogenous ATP level in lysed chloroplasts. Although this treatment enhanced the ATP dependency, it also resulted in a loss of conjugation, especially to proteins in the high mol wt range (Fig. 7). We concluded, based on these observations, that neither preincubation nor gel filtration could be used routinely to decrease ATP levels in the plants.

Another feature of conjugation in crude green leaf extracts was that we usually found increasing conjugation in assays for more than 60 min, suggesting perhaps less proteolysis of conjugates in the chlorophyllous extracts than in previously recorded etiolated ones (Figs. 1 and 6; ref. 27). This may be supported by the lack of response to the protease inhibitor leupeptin. Vierstra (27) and Haas *et al.* (8) found significant enhancement of recorded conjugation in the presence of this inhibitor. This was not the case in green tissue, which may be attributed to the fact that our assays were performed at pH 8.2, and that the proteolytic inactivation was found to be very pH dependent (8). However, we did get some enhancement of conjugation with the inhibitor APMSF (data not shown), suggesting that there might be some breakdown of ubiquitin and conjugating enzymes with time. In agreement with this, extending the assay over more than 3 h usually resulted in loss of conjugates.

In summary, our results show that light-grown oat plants contain the ubiquitin-dependent proteolytic system. In green leaves, chloroplasts are able to ubiquitinate proteins, and we have identified RuBPCase as a possible substrate. The presence of ubiquitin in chloroplasts of *Chlamydomonas* has recently been shown by immunogold labeling (31). One criterion for demonstrating ubiquitin-dependent conjugation in plants is to prove ATP-dependence. However, in green tissues, the ATP level is much higher than that needed for conjugation. Preincubation under ATP-depleting conditions was only succesful when isolated chloroplasts were used. The pH optimum of 8.2 suggests that it is unlikely that conjugating enzymes are active in the plant vacuole. Therefore, unless ubiquitin-protein conjugates are transported into the vacuole, vacuolar proteases may not be involved in any ubiquitin-dependent proteolysis.

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