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Genetics

Use of a reporter transgene to generate *Arabidopsis* mutants in ubiquitin-dependent protein degradation

(degradation signal/methotrexate resistance/N-end rule)

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ABSTRACT **Ubiquitin-dependent proteolysis is a major proteolytic pathway in the cytoplasm and nucleus of eukaryotic cells. We introduced a gene encoding a substrate for this pathway into the genome of *Arabidopsis thaliana*. The transgene codes for a hybrid protein consisting of dihydrofolate reductase (DHFR, EC 1.5.1.3) fused to a degradation signal that is specifically recognized by components of the ubiquitin-dependent proteolysis pathway. Elevated concentrations of the DHFR protein confer resistance to the drug methotrexate, but rapid degradation prevents accumulation of the protein in the plant. Therefore, transgenic *A. thaliana* lines expressing the DHFR fusion protein are methotrexate-sensitive. Selection for mutants resistant to methotrexate resulted in plants impaired in degradation of the DHFR model substrate, as shown by an increase in protein level in the mutants.**

In every eukaryotic cell, a set of enzymes is capable of posttranslationally ligating the small protein ubiquitin to a variety of cellular proteins (1–5). The enzyme E1 (ubiquitin-activating enzyme) forms a thiol ester bond with the carboxyl-terminal glycine residue of ubiquitin. This activated ubiquitin moiety is transferred to a member of the E2 enzyme family (ubiquitin-conjugating enzymes). E2 enzymes can, either alone or in concert with additional factors (E3 proteins; ubiquitin protein ligases), catalyze coupling of the carboxyl terminus of ubiquitin to ε-amino groups of lysine residues in a substrate protein.

This reaction has been shown to be involved in functions as diverse as cell cycle control, DNA repair, and proteolysis of naturally short-lived and abnormal proteins.

Although some ubiquitination events involve addition of one ubiquitin residue to a target protein, ubiquitin-dependent proteolysis generally requires stepwise addition of several ubiquitin moieties. The first ubiquitin is linked directly to the substrate, whereas every additional ubiquitin moiety is joined to the previously attached ubiquitin to form a "multiubiquitin" chain (6). Thereafter, such branched proteins are substrates for a large multicatalytic protease, the 26S proteasome, which releases ubiquitin for reutilization and generates short peptides as degradation products of the substrate protein (7–9). The whole process requires metabolic energy in the form of ATP.

Selection of target proteins by the ubiquitin-dependent proteolysis system involves recognition of specific domains of proteins. These domains can either bind directly to components of a ubiquitinating enzyme complex or are subject to prior modification, with degradation being a consequence of other cellular events.

So far, two degradation signals have been characterized in more detail. One, the "destruction box" of G_2 cyclins, mediates degradation at only one specific point in the cell cycle (10). The other, called N-end rule degradation signal (or N-degron; ref. 11), is apparently constitutive and universal in all eukaryotes studied (5, 12, 13), including tobacco plants (F.B., J.S., and A.B., unpublished data).

To learn more about the role of ubiquitin-dependent proteolysis in plants, we have been studying its function *in vivo* by using a transgenic tobacco system (14). As a complementary approach, we also wanted to apply the tools of classical and molecular genetics and, therefore, used the small crucifer *Arabidopsis thaliana*, generally considered a model plant for such studies, to generate mutants in ubiquitin-dependent proteolysis. Here, we describe the strategy used to obtain these mutants and present a genetic and biochemical analysis. One complementation group obtained, designated *prt1*, defines a single nuclear gene whose recessive mutational change results in a decreased or completely abolished ability to degrade a model substrate for ubiquitin-dependent proteolysis.

MATERIALS AND METHODS

Plants, Microbial Strains, and Vectors. *A. thaliana* ecotype Col-0 was used for plant experiments. *Escherichia coli* MC 1061 (15) was used for cloning work. *Agrobacterium tumefaciens* GV 3101 pMP90RK (16) was used for *Agrobacterium*-mediated plant transformation. Vector pPCV-501-3′Hyg (16) was a gift of C. Koncz (Max-Planck-Institut, Cologne). The dihydrofolate reductase (DHFR; EC 1.5.1.3)-containing construct XVI has been described (12).

Vector Construction. A plant transformation vector (pCHUM) was assembled from plasmids pPCV-501-3′Hyg (16), pRT103 (17), construct XVI (12), and pRTUB1 [a plasmid analogous to pRTUB32 (14) but with one repeat of plant ubiquitin] by using standard techniques (15).

In its final form, the assembled gene is under the control of the nopaline synthase promoter (from pPCV-501-3′Hyg) and the cauliflower mosaic virus 35S RNA terminator (from pRT103). The open reading frame is from construct XVI, except that yeast ubiquitin was replaced with plant ubiquitin (from pRTUB1) and that phenylalanine is at the ubiquitin–DHFR junction. The nopaline synthase promoter of pPCV-501-3′Hyg was replaced by the cauliflower mosaic virus 35S promoter (from pRT103), and the DHFR construct was inserted at the *Hin*dIII site of this modified vector. Details of the cloning protocol can be obtained upon request.

Plant Transformation. Plant transformation was carried out essentially as described (18) using hygromycin resistance mediated by pCHUM as a selectable marker in *A. thaliana* ecotype Col-0.

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate.

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Mutagenesis and Mutant Detection. Two independently transformed *Arabidopsis* lines were mutagenized with ethyl methanesulfonate by standard methods (19) using 0.1% ethyl methanesulfonate (Sigma) for 10 h. M_2 seeds were harvested from 12 batches, each of ≈2000 M_1 plants. Sterilized seeds were plated on plant agar medium [MS salts (Imperial Laboratories, U.K.; 4.6 g/liter)/3% (wt/vol) sucrose/0.9% agar (Merck) supplemented with thiamine (10 mg/liter), biotin (0.2 mg/liter), pyridoxine (1 mg/liter), nicotinic acid (1 mg/liter), *myo*-inositol (0.1 g/liter), and 2-(*N*-morpholino)ethanesulfonic acid (0.5 g/liter, pH 5.8) containing hygromycin (20 mg/liter) and methotrexate (MTX; 0.1 mg/liter)]. Plantlets able to develop roots in the agar were scored as positive. For genetic analysis, mutants were crossed to one reference mutant (allele *prt1-1*) and to the nonmutagenized transgenic line (outcross). Both F_1 and F_2 progeny from the outcross were tested on MTX plates to assess recessiveness of the alleles obtained.

RNA Analysis. Plant RNA was prepared with oligo(dT) beads (Dynal, Skøyen Norway) by using the manufacturer's recommendations. Gel preparation, transfer onto nylon membrane (Amersham; Hybond-N), and hybridization were done as described (15). Equal loading of poly(A)$^+$ RNA was assessed by hybridizing the blot with ch-42 cDNA (20).

***In Vivo* Labeling and Immunoprecipitation.** Leaf material was from 15 small rosette leaves each of which was cut into four pieces and put into 0.5× MS medium (i.e., medium as described for agar plates but without agar and diluted 1:1 with water). After addition of 500 μCi of [^{35}S]methionine (Amersham; 1 Ci = 37 GBq), the sample was mildly evacuated for two 5-min periods to fill intercellular spaces and further incubated for 3 h at 20°C. Care was taken to keep the samples covered with liquid (rotory movement). After three washes with the above medium containing 1% methionine, glass beads (<106 μm; Sigma) and pestle were used to homogenize the leaf material in extraction buffer [2% (wt/vol) SDS/30 mM dithiothreitol/90 mM sodium Hepes, pH 7.5]. After 5 min at 100°C, insoluble material was centrifuged down, reextracted with the same buffer, and pelleted by centrifugation again. The combined supernatants were used to determine radioactivity incorporated into protein as described (12). Samples containing 8 × 10^6 cpm were diluted 1:10 with buffer B (12). Protein A-Sepharose (Pharmacia; 20 μl) was added for a 20-min incubation and removed by centrifugation. Thereafter, affinity-purified rabbit anti-mouse DHFR antibody was added and the procedure was continued as described (12). Electrophoresis and fluorography were done as described (12) using 10 × 5 cm minigels (Biometra, Göttingen, F.R.G. BRD).

RESULTS

A Strategy to Isolate Mutants in Ubiquitin-Dependent Protein Degradation in *A. thaliana* Based on Changes in Metabolic Stability of a Reporter Protein. Wild-type seedlings of *A. thaliana* die at a very early stage when the seeds are germinated on agar medium containing MTX at 0.1 mg/liter. Most of them are barely able to project a root from the seed coat. Introduction of a gene coding for murine DHFR with reduced affinity for MTX, driven by a plant promoter, confers resistance to *Arabidopsis* plants (21).

We made use of a modified MTX resistance gene to identify and isolate mutants with lesions in ubiquitin-dependent protein degradation by the scheme depicted in Fig. 1*A*. A DHFR protein that is short-lived because it contains a degradation signal is expected to lead to increased MTX resistance only if the degradation machinery is inactivated by a mutation.

To serve as a degradation signal, we used a sequence that was extensively characterized in the yeast *Saccharomyces cerevisiae* (12) and was subsequently shown to work as a signal for ubiquitin-dependent proteolysis in mammals, as well (13). Experiments in tobacco indicated that the same sequence is also conferring a short half-life to proteins in plants (unpublished results).

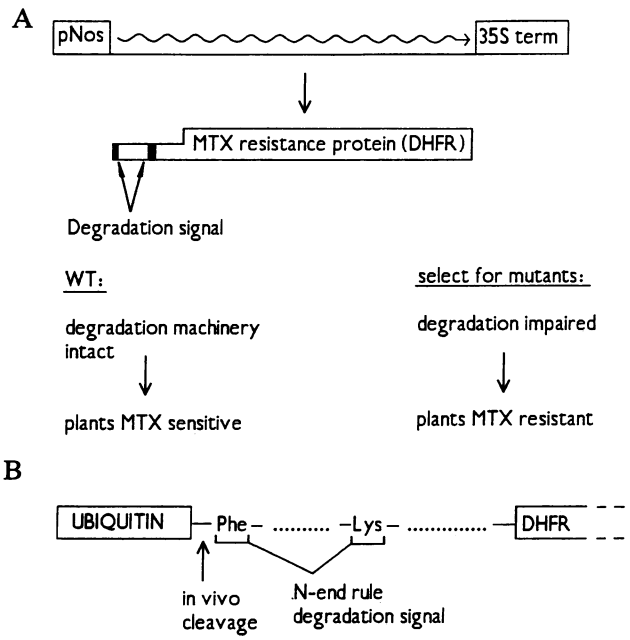

FIG. 1. Selection of *Arabidopsis* mutants deficient in ubiquitin-dependent proteolysis. (*A*) A transgene encodes a DHFR protein with amino-terminal extension mediating rapid degradation. (*B*) Schematic representation of the degradation signal consisting of the first residue, phenylalanine, and an internal lysine residue (cf. ref. 12). pNos, nopaline synthase promoter; 35S term, terminator of cauliflower mosaic virus 35S RNA; WT, wild type.

Fig. 1*B* shows the relevant features of this N-end rule degradation domain. It consists essentially of the first amino acid that has to contain a bulky side chain (in our case the phenyl group of phenylalanine) and at least one internal lysine residue that has to be easily accessible to the ubiquitinating complex (12). To generate an exposed bulky first residue, we made use of the fact that proteases capable of hydrolyzing linkages between ubiquitin and substrate proteins occur in all eukaryotes and cleave translational fusions of ubiquitin to other proteins precisely at the junction. (The natural role of these enzymes lies in diverse deubiquitination reactions; Met-aminopeptidase, the enzyme involved in normal amino-terminal processing, does not remove methionine if the second residue has a bulky side chain.) Thus, immediately after or during translation, ubiquitin is cleaved off the fusion protein by a ubiquitin-specific protease, generating the degradation domain as a part of the hybrid DHFR protein.

We constructed a vector containing the gene depicted in Fig. 1*A*, in which a hybrid DHFR protein is expressed under control of the relatively weak napoline synthase promoter. Although the amino-terminal extension as such does not interfere with enzymatic activity of the DHFR protein, rapid degradation should lead to a steady-state level too low for MTX resistance to emerge in transformed plants.

This gene was introduced into the genome of *A. thaliana*. Several lines obtained showed resistance to hygromycin (the selective marker for transformation) but did not exhibit marked resistance to MTX (Fig. 2, seedlings in the two bottom rows). In general, seedlings containing the transgene developed to a slightly later stage than untransformed ones (data not shown), but roots did not grow in agar containing MTX in either case. This observation is consistent with the hypothesis that the "MTX resistance gene" did not confer

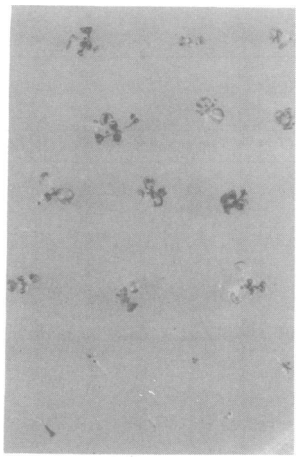

FIG. 2. *A. thaliana* seeds germinated on MTX-containing agar. *prt1-1* mutant plants (upper four rows) can grow in presence of MTX, whereas *PRT1* wild-type seedlings cannot (lower two rows). All plants contain the transgene shown in Fig. 1.

resistance because of the short half-life of the hybrid DHFR protein.

Mutants of Transgenic *A. thaliana* Expressing Short-Lived DHFR Selected for Ability to Grow in the Presence of MTX. After mutagenesis, 12 groups of M_1 plants (each from ≈2000 mutagenized seeds) were grown to maturity. M_2 seeds were germinated on Petri dishes containing MTX in the agar medium. At the density chosen (≈200 seeds per 10-cm plate), many seedlings could expand the cotyledons. However, among the ≈60,000 seeds tested, only a dozen seedlings were able to form roots in MTX-containing agar. We considered these plantlets as potential mutants and used them for further analysis.

Crosses to one reference mutant showed that 6 of the 12 mutants, obtained from four M_1 pools, fell into one complementation group designated *prt1* (*prot*eolysis). Fig. 2 shows a comparison of homozygous *prt1-1* mutant seedlings (four upper rows) with the unmutagenized transformed line (two lower rows). There was a clear difference in the ability to grow in the presence of MTX between the transformed line and *prt1-1* mutant plantlets. Table 1 shows the result of an outcross of the *prt1-1* mutant to the unmutagenized transformed line. Although we cannot rule out minor changes in half-life of the reporter gene in heterozygous plants, the numbers and phenotypic classes obtained from analysis of the cross between *prt1-1* and *PRT1* plants (Table 1) are most consistent with a single nuclear recessive mutation.

Finally, we wanted to look at mRNA and protein levels in mutant vs. wild type. Fig. 3 shows the result of a Northern blot analysis. A control hybridization was carried out with a probe from the *ch-42* locus (ref. 20; Fig. 3*B*). Both *prt1-1* and *PRT1* plants contained the same amount of mRNA transcribed from the transgene (Fig. 3*A*). We conclude that the mutation does not affect mRNA levels.

This situation contrasts with a striking difference in the level of DHFR protein. As shown in Fig. 4, *in vivo* labeling

Table 1. *prt1-1* is a recessive mutation in a single nuclear gene

Seeds, no.		Seedlings, no.	
Total tested	Not germinated	Capable of forming roots in MTX agar	Growing poorly in presence of MTX
1186	52	280	854

Progeny of seven individual F_1 plants homozygous for the transgene and heterozygous for the *prt1-1* allele was tested. The χ^2 result for the hypothesis of a 3:1 segregation is 0.042 ($P \approx 0.84$).

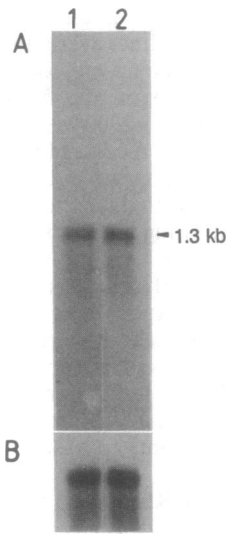

FIG. 3. Northern blot analysis of transgene mRNA. No difference between wild type (lane 1) and *prt1-1* mutant (lane 2) can be detected. (*A*) Hybridization of poly(A)$^+$ RNA from *A. thaliana* with the DHFR gene. (*B*) Same as *A* but probe was ch-42 cDNA (20) as an internal standard. kb, kilobases.

of leaf proteins followed by immunoprecipitation, SDS/PAGE, and fluorography allowed us to detect DHFR in the *prt1-1* mutant but not in the wild-type genetic background. Thus, we found an increase in the steady-state level, leading to the conclusion that the *prt1-1* mutation increases the half-life of the otherwise short-lived transgene product. The fact that the protein appeared as a doublet may be due either to an *in vivo* cleavage within the extension peptide by a cytoplasmic protease or to cleavage *in vitro* upon cell disruption. Interestingly, the same pattern occurred in the tobacco control lane (Fig. 4, lane 3), and a similar situation has also been found in yeast (12).

DISCUSSION

We describe the generation of mutants of *A. thaliana* with deficiency in protein degradation. To isolate these mutants,

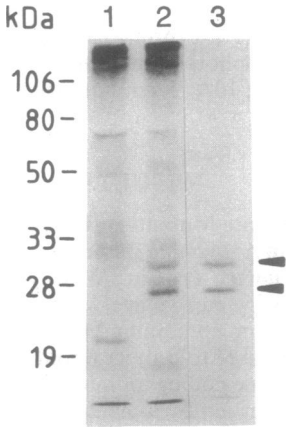

FIG. 4. Increased DHFR protein level in *prt1-1* mutant. Leaf proteins were labeled with [^{35}S]methionine, extracted, and used for immunoprecipitation followed by SDS/PAGE and fluorography. In *Arabidopsis* wild-type leaves (lane 1), no specific bands can be detected. In the *prt1-1* mutant (lane 2), a doublet is precipitated that comigrates with the marker protein isolated from transgenic tobacco leaves by the same method (lane 3). The marker protein is identical with the one in lane 2 except for methionine at position 1 to inactivate the degradation signal. The lower band probably results from a proteolytic cut unrelated to ubiquitin-dependent proteolysis.

we extended a DHFR protein with a stretch of amino acids that is a degradation signal for ubiquitin-dependent proteolysis and thus confers a short half-life onto the hybrid protein. Although introduction of a gene encoding the unmodified DHFR protein leads to MTX resistance in *A. thaliana*, expression of the extended protein leaves the plants sensitive to the drug. Selection for resistance among a mutagenized seed population resulted in isolation of mutants that were analyzed genetically and by investigation of transgene mRNA and protein levels.

Genetic changes leading to MTX resistance have been obtained previously in mammalian cell culture systems. They were either specific for the drug MTX, resulting from amplification of the DHFR gene (22), or they resulted in resistance to a variety of other drugs, as well (known as multiple drug resistance; ref. 23).

Our mutants do not fall into either class, because they are recessive. We can also rule out the occurrence of a promoter-up mutation in the transgene, because the resistance phenotype in the mutant is not accompanied by an increase in mRNA abundance.

In striking contrast, the steady-state level of the hybrid DHFR protein is increased at least 7-fold in one of our mutants (Fig. 4 and data not shown). This points to mutations that interfere with degradation of the protein. In particular, mutations in the degradation signal that make the signal less efficient and in the degradation machinery would increase the half-life of the reporter protein. The target site for mutations of the former type, however, is only one or two nucleotides, because a change in the degradation signal would have to affect the first amino acid, phenylalanine [see Fig. 1*B*; the internal lysine residue is redundant in the sequence chosen (12)]. Furthermore, such a change would result in a dominant mutation, which is at variance with the experimental evidence showing that the alleles obtained are recessive. Thus, we believe that the mutations detected by our reporter gene affect the degradation machinery.

From the experiments presented here, it seems likely that analogous screens can be carried out using additional distinct degradation signals and thus inactivating ubiquitinating complexes (i.e., ubiquitin–protein ligases and ubiquitin-conjugating enzymes) specific for other degradation signals.

The great difference in DHFR protein levels between *prt1-1* mutant and wild type (see Fig. 4) suggests the use of this mutant in lieu of an inducible promoter. Any protein of choice that tolerates amino-terminal extensions can be coupled to the N-end rule degradation signal for rapid degradation in the wild-type background. In the mutant background, the protein is "induced" by abolishing degradation (cf. refs. 5 and 24).

It is interesting to compare our mutants with the situation in the budding yeast *S. cerevisiae*. A number of mutations are known in components of ubiquitin-dependent proteolysis that abolish degradation of a substrate protein consisting of β-galactosidase as a reporter gene with the same degradation signal used in our experiments. Two mutations affect components of the ubiquitinating complex (25, 26), and two mutations affect the downstream multicatalytic protease, the proteasome (27, 28). As the components of the ubiquitin system are well conserved (29), the existing knowledge should help to identify the genes affected in our mutants.

Conservation of components of the system leaves open the possibility that, in plants, ubiquitin-dependent proteolysis serves functions specific to particular tissues or stages of the life cycle. Complementary work using a transgenic tobacco model (14) suggests that this is indeed the case.

Preliminary examination of phenotypic consequences of the *prt1-1* mutation suggests no dramatic effects during normal growth. There is a slight delay in seed germination on earth and possibly a generally slower life cycle. However, more dramatic effects may show up during a variety of stress conditions or in double mutants.

Thus, the *Arabidopsis* mutants in ubiquitin-dependent protein degradation can be used to investigate the role of this pathway in a plant's life cycle and can be subjected to a detailed physiological characterization.

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