Chapter 5

Fluorescent Reporters for Ubiquitin-Dependent Proteolysis in Plants

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Abstract

Ubiquitin is a small protein commonly used as a signal molecule which upon attachment to the proteins affects their function and their fate in the cells. For example, it can be used as a degradation marker by the cell. Ubiquitin plays a significant role in regulation of numerous cellular processes. Therefore, monitoring of ubiquitin-dependent proteolysis can provide important information. Here, we describe construction of YFP-based proteasome substrates containing modified ubiquitin and the protocol for their transient expression in plant cells for functional analysis of the ubiquitin/proteasome system. To facilitate further subcloning all plasmids generated by us are based on the Gateway® Cloning Technology and are compatible with the Gateway® destination vectors.

Key words Fluorescent reporters for ubiquitin, UFD, UPS

1 Introduction

There are two major degradation machineries in plants, ubiquitin–proteasome system (UPS) and autophagy pathway. UPS functions in two cellular compartments, the cytoplasm and the nucleus, and requires 26S protein complex (proteasome) for protein clearance. Contrary to UPS, autophagy operates only in the cytoplasm and involves vesicle transport to deliver various cellular components to be degraded in the vacuole. Both pathways may use a small highly conserved 76-aa protein, called ubiquitin (Ub), to mark substrates for degradation. Despite that ubiquitination is an important determinant of autophagy selectivity and that autophagy can take over degradation of the ubiquitin–proteasome pathway substrates when UPS is impaired [1] ubiquitination is not critical in autophagy and several ubiquitin-independent selective autophagy receptors has been already described [2]. In contrary, ubiquitination is crucial for tagging short-lived soluble proteins for degradation by UPS. It is worth to mention that it is UPS which is mainly responsible for regulated and progressive degradation of such intracellular proteins.

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Ubiquitination of proteins designated for UPS degradation occurs though a three-step sequential action of E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme), and E3 (Ub ligase) enzymes [3]. Proteins are usually modified by more than one Ub molecule. The sequential Ub ligation to another Ub molecule previously attached to the protein causes elongation of the Ub chain (polyubiquitination). The polyubiquitinated proteins, especially those marked by a K48 chain, are main substrates for proteasomal degradation. In eukaryotes, the N-end rule pathway and the Ub-fusion degradation (UFD) pathway are a part of the UPS and they regulate half-life of many proteins. The ubiquitination pathways for the N-end rule and UFD pathways have been mapped in detail. It is known that both pathways do not overlap and possess diverse degradation signals, as well as require different E2–4 factors, chaperons or ubiquitin-binding subunits involved in proteasomal targeting of each of the substrates. The UFD substrates are ubiquitinated within the N-terminal ubiquitin moiety. The UFD pathway recognizes these "nonremovable" N-terminal Ub moiety as a primary degron, whereas N-end rule substrates before ubiquitination on the substrate itself require cleavage of the N-terminal ubiquitin by isopeptidases.

Monitoring of Ub-dependent proteolysis is very important because of the requirement for Ub in both protein degradation pathways (UPS and autophagy) and its significant role in regulation of numerous cellular processes. As a matter of fact, it has been already shown that the production of GFP-based proteasome substrates by fusion to the N-terminus of GFP-specific degrons for N-rule or for UFD pathways successfully allow to perform functional analysis of the UPS and to monitor the cross-talk between UPS and autophagy in mammals and yeast [4, 5]. Here, we report the protocol for obtaining similar fluorescent reporters for ubiquitin-dependent proteolysis in plants. To investigate the functionality of these fluorescent reporters in plants, the plant expression cassettes encoding the stable (Ub-M-YFP) and the UFD substrate (Ub^{G76V}-YFP) fusion proteins were created under the 35S promoter in the appropriate binary plasmids. As expected, microscopy and western blot analysis of transiently transformed *N. benthamiana* epidermis expressing these Ub-X-YFP fusions showed stability of Ub-M-YFP while the expression of Ub^{G76V}-YFP resulted in low fluorescent intensity confirmed by western blot.

2 Materials

2.1 PCR and Plasmid Recombination

Prepare all solutions using ultra-pure sterile water freshly before use and keep them on ice. Grow *Escherichia coli* strains at 37 °C and *Agrobacterium tumefaciens* at 28 °C. Shake liquid cultures at 150–300 rpm in a rotary shaker.

1. Standard *E. coli* strains used for cloning and plasmid amplification or for recombinant protein expression should be grown in conventional bacterial growth media LB (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl with pH adjusted to 7 with NaOH and additionally 15 g/l agar for LB plates) and SOB (20 g/l tryptone, 5 g/l yeast extract, and 0.5 g/l NaCl, 0.186 g/l KCl with pH adjusted to 7 by adding NaOH) [6].
2. Add antibiotics to the media: kanamycin (stock solution: kan, 50 mg/ml in water, final concentration: kan, 50 mg/l) for pENTR/D-TOPO vector carrying bacteria, chloramphenicol (stock solution: can, 30 mg/ml in ethanol, final concentration: cam, 30 mg/l) plus streptomycin (stock solution: sp, 50 mg/ml in water, final concentration: sp, 50 mg/l) for bacteria containing donor vector such as pH7YWG2 and streptomycin (sp, 50 mg/l) for bacteria carrying destination (binary) plasmid.
3. *A. tumefaciens* strain LBA4404 should be grown in YEB medium (5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 5 g/l sucrose, 0.5 g/l MgCl2, and additionally 15 g/l agar for YEB plates) [7] with rifampicin (stock solution: rif, 30 mg/ml in chloroform, final concentration: rif, 30 mg/l) plus streptomycin (sp, 75 mg/l).
4. Use primers listed below for amplifying cDNA for tobacco *Ub* (*see* **Note 1**):

   Forward (F): 5′-CACCATGCAGATCTTCGTGAAGACATTGAC-3′

   Reverse (R1): 5′-CTTACCCATACCACCACGGAGACGGAGGAC-3′

   Reverse (R2): 5′-CTTACCAAC**AAC**ACCACGGAGACGGAGGAC-3′

   The reverse primer R1 is designed to amplify full length ubiquitin with additional linker at the C-terminus coding MGK tripeptide (underlined).

   The reverse primer (R2) is designed to generate G76V substitution in Ub (bold) and to add additional linker (VGK tripeptide) at the C-terminus (underlined).
5. pENTR/D-TOPO cloning kit (Life Technologies, cat. number K2400-20).
6. LR recombination kit (Life Technologies, cat. numbers 11791-043 or 11791-020).
7. PCR reagents including polymerase and dNTPs (for example, Life Technologies Pfu polymerase, cat. number EP0502 and Life Technologies dNTP set, cat. number 10297-018).
8. The pH7YWG2 binary plasmid can be ordered on line from http://www.vib.be/en/research/services/Pages/Gateway-Services.aspx.

2.2 Transient Transformation

1. Cultivate *Nicotiana benthamiana* plants in soil in a growth chamber under the conditions of 60% relative humidity, with a day/night regime of 18 h light 300 μmol photons/m^2/s at 21 °C and 6 h dark at 18 °C, or in a greenhouse till they fully expanded leaves achieved about 5–6 cm in diameter approx. 4–5 weeks (*see* **Note 2**).
2. Prepare four 10-ml syringes without needles, two microscopic cover slides and cover slips.

2.3 SDS-PAGE and Western Blot

1. Use either the precast SDS-PAGE gels (for example, 12% Mini-PROTEAN TGX, Bio-Rad, cat. number 456-1041) or the fresh 12% SDS-PAGE gels prepared according to the published protocols [6].
2. Extraction Buffer: 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% β-mercaptoethanol, 0.0005% PMSF.
3. Bio-Rad protein assay kit (cat number 500-0002).
4. 4× Laemli Sample Buffer: 200 mM Tris–HCl pH 6.8, 8% SDS, 40% glycerol, 50 mM DTT, 0.02% bromophenol blue. Store at 4 °C.
5. 1× Running Buffer: 25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3.
6. 1× Transfer Buffer: 25 mM Tris base, 0.192 M glycine.
7. Blocking Solution: 5% dried milk in PBS. Store at 4 °C.
8. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.

3 Methods

3.1 Plasmid Creation

1. To amplify cDNA encoding ubiquitin from organism of choice (in this case tobacco) prepare fresh cDNA. Extract total plant RNA from frozen powdered material using, for example, the cold phenol method [8] (*see* **Note 3**) and subsequently use purified total RNA as templates for reverse transcription-polymerase reaction as described in **Note 4**.
2. Prepare 50 μl PCR mix solutions on ice. Mix the appropriate primers (F and R1 in one reaction; F and R2 in the second reaction) at 0.1–0.5 μM each with 1× PCR reaction buffer containing ~0.2 mM dNTPs and 1 U of Pfu polymerase. Finally add about 200 ng of plant cDNA to amplify and incorporate appropriate mutations into *Ub* cDNA.
3. Use following PCR reaction parameters: initial denaturation at 94–95 °C for 2 min, 30 cycles: 30 s at 94 °C (denaturation), 30 s at 62 °C (an annealing temperature) and elongation at 72 °C for 30 s, and a final 5-min finishing elongation at 72 °C.

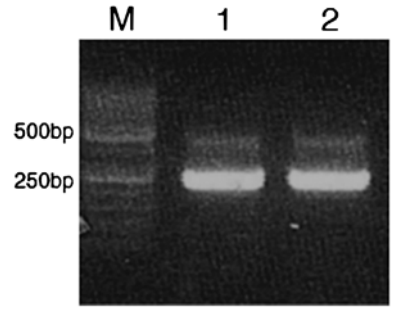

Fig. 1 Electrophoretogram (2 % agarose) of PCR products. PCR products derived from F + R1 or F + R2 primer sets used to amplify Ub-M and to Ub^{G76V}, respectively, were separated using a 2 % agarose gel in TAE buffer. *Lane M*: GeneRuler 50 bp DNA Ladder from Life Technologies, *lanes 1* and *2*: Ub-M and to Ub^{G76V}, respectively

4. Resolve PCR products on agarose gels (2 % in 1× TAE buffer) according to the standard procedure [6]. To visualize DNA bands in UV light ethidium bromide can be added to the gel to 0.2 μg/ml prior to pouring.
5. Excise the appropriate bands from the gel and purify using any gel extraction kit (Fig. 1).
6. Independently clone both PCR products into the pENTR/D--TOPO vector according to manufacturer's protocol and select positive colonies by sequencing.
7. Finally, create destination vector by LR recombination reaction using pH7YWG2 plasmid according to the manufacturer's procedure (*see* **Note 5**).
8. Analyze colonies by sequencing to verify proper insert incorporation into the open reading frame. The linker sequence between Ub and fluorescent protein should be as it is described on Fig. 2.
9. Transform independently *A. tumefacien*s by each of two destination plasmids using either the high-voltage electroporation or the heat shock method (*see* **Note 6**). After transformation, add 0.5 ml of YEB, incubate cells for 3–4 h at 28 °C in a water bath, spread mixture on the appropriate selective plates and incubate at 28 °C for 3–5 days.

3.2 Transient *N. benthamiana* Leaf Transformation

1. Cultivate *N. benthamiana* plants till fully expanded leaves achieve about 5–6 cm in diameter.
2. When young *N. benthamiana* plants are ready prepare 3 ml fresh overnight cultures of *A. tumefaciens* strains containing the appropriate destination (binary) plasmids.
3. Spin cells down, and wash twice in sterile ddH_2O to remove medium.

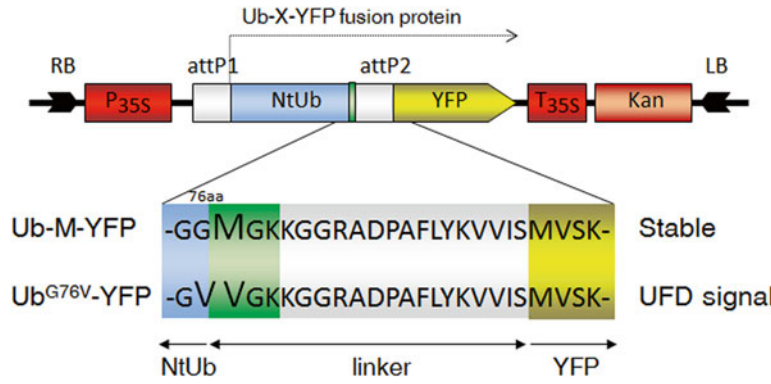

Fig. 2 Schematic representation of the T-DNA cassettes containing Ub-X-YFP fusion proteins. The amino acid sequence of the ubiquitin is shown in *blue*, the linker sequence is shown in *green* (inserted amino acids during PCR reaction) and in *gray* (residues corresponding to attP2 region) and the YFP sequence is highlighted in *yellow*. The glycine residue in position −1 of Ub^{G76V}-YFP is substituted by valine

4. Re-suspend each cell suspensions in 5–10 ml ddH_2O to obtain a final cell density of about $1–6 \times 10^8$ cells/ml (OD_{600} from 0.1 to 0.6).
5. Inoculate one half of the leaf (the lower epidermis) with the prepared suspension of *A. tumefaciens* containing the plasmid enabling expression of the stable fluorescent protein (Ub-M-YFP) and the second half of the leaf with the second suspension (for expression of the unstable fluorescent protein Ub^{G76V}-YFP) using needle-less syringes by placing the syringe against the underside of the leaves and gentle pressing. Leave half a centimeter borders near the leaf nerve to prevent the transfer of bacteria (Fig. 3).
6. After 2–3 days of incubation harvest the leaf for transgene expression analysis by confocal microscope and western blot.

3.3 Microscopy Observation

1. On the third day post-agroinfiltration, cut the scrape (for example, 1×1 cm square) from the center of each leaf half with a sharp scalpel. Collect the rest of plant material for western blot analysis.
2. Immediately place each scrap into the separate 10-ml syringe without needle, cover by your finger (protected by laboratory gloves) the bottom opening of the syringe and fill it with 5 ml of water. Remove your finger from the bottom of the syringe and pull the air. One more time use your finger to cover the bottom of the syringe and pull the plunger to create a vacuum. You should see the air bubbles coming from the leaf scrap. Release plunger. The leaf scrap should become dark green due to flooding the intercellular air spaces with water.

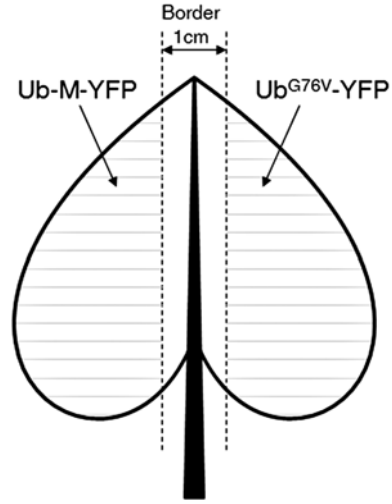

Fig. 3 Scheme of the leaf lower epidermis division into two halves for two simultaneous agroinfiltrations. The safe border preventing bacterial penetration of second leaf half is marked

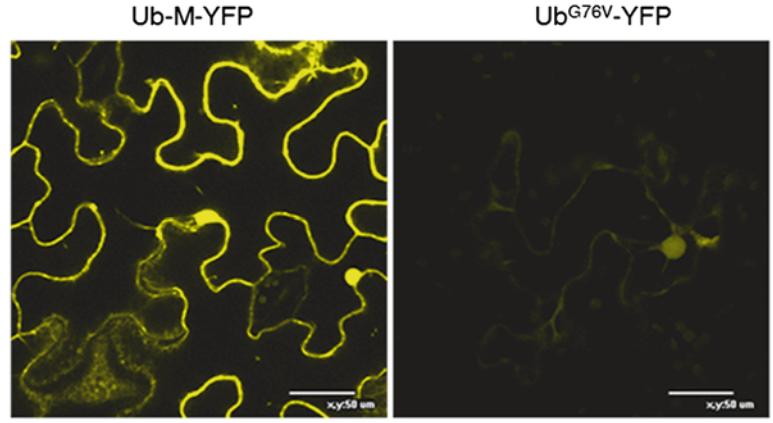

Fig. 4 Representative images of the expression level of the Ub-X-YFP fusion proteins in *N. benthamiana*. Stability of the YFP chimeras was analyzed 3d post-agroinfiltration using fluorescent confocal microscope by determining intensity of the YFP fluorescence under the same set of the parameters

3. Take out leaf scraps from the syringe and prepare microscopic slides (*see* **Note** 7).
4. Analyze each scrap in the fluorescent confocal microscope under the same set of the parameters (Fig. 4).

3.4 Western Blot Analysis

1. Third day post-agroinfiltration collect each half of the leaf tissue into the Eppendorf tube (approximately 100 mg) for western blot analysis. Freeze the plant material in liquid nitrogen.
2. Grind tissue in 100 μl of Extraction Buffer to extract proteins.

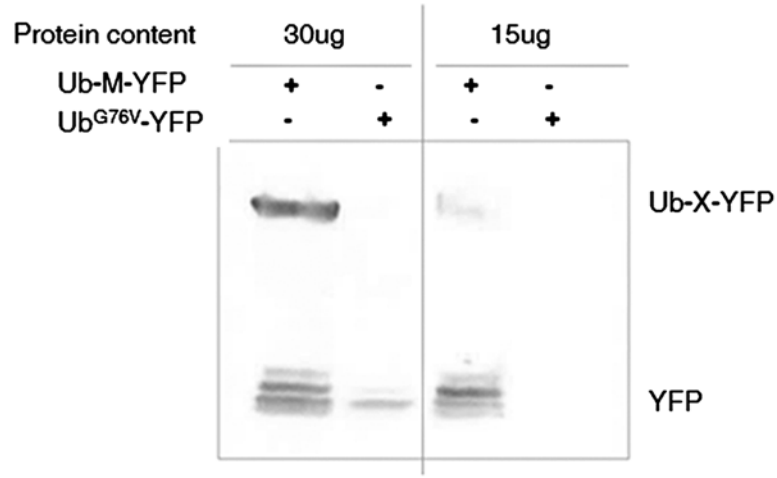

Fig. 5 Western blot indicating the amount of the Ub-X-YFP fusion proteins. Expression of the YFP chimeras was analyzed 3d post-agroinfiltration by western blot with an anti-GFP antibody. The uncleaved precursors and polypeptides with sizes corresponding to the cleaved degraded products are indicated as Ub-X-YFP and YFP, respectively

3. Shake the tubes in the thermomixer for 5 min and spin 10 min in a microcentrifuge at 10 rpm in a cold room.
4. Collect the supernatants in the new Eppendorf tubes and determine the concentration of the recombinant proteins using a standard method, for example, with the Bio-Rad protein assay kit.
5. Take a volume with a given protein amount (usually 15–30 μg), add 1/3 of the volume of 4× Laemli Sample Buffer and denature by boiling for 5 min. Subsequently cool on ice and spin briefly.
6. Load samples into 12% SDS-polyacrylamide gel and run the gel in reducing conditions [6] using conditions recommended by the manufacturer of the electrophoresis apparatus.
7. Perform the western blot analysis according to a standard procedure [6] using anti-GFP antibody (Fig. 5).

4 Notes

1. To create the substrate for N-end rule pathway which will be compatible with described above fluorescent reporters one can introduce in reverse primer R1 a single point mutation to form one more linker (RGK tripeptide) at the C-terminus. The lysine residues in position 3, 4, and 15 in the linker between ubiquitin and the fluorescent protein (downstream of the ubiquitin moiety) will be potential ubiquitination sites *for* ubiquitin ligase of the N-end rule pathway.

2. *N. benthamiana* plants can be alternatively cultivated hydroponically in hydroponic containers filled by 0.5× Hoagland's nutrient solution. Hoagland's media should be buffered with 2 mM (0.39 g/l) MES (2-[*N*-Morpholino]ethanesulfonic acid) and adjusted to pH 5.5 with 1 M KOH before autoclaving (for 15 min at 121 °C).
3. Add 1 ml TRI REAGENT® (Sigma-Aldrich) to 50–100 mg of homogenized plant tissue, vortex, and keep 5 min at room temperature. After 10 min centrifugation at 12,000 g transfer the supernatant to a new microfuge tube and add 100 μl of bromochloropropane, vortex 15 s, and keep 2–15 min at room temperature. Spin 12,000 g for 15 min at 4 °C, collect the upper phase (~600 μl) to a new microfuge tube and add 500 μl of isopropanol for RNA precipitation. Then vortexed the samples and after 2–15 min on ice, spin 12,000 g for 8 min (cold or room temperature), discard the supernatant, add 1 ml of 70% ethanol and mix (RNA wash). Spin 7500 g for 5 min to remove the ethanol and leave the pelleted RNA to air dry for 2–3 min. Finally, add 10–20 μl of TE pH 8 and resuspend RNA by pipetting. Alternatively, for total RNA isolation use DirectZol™ RNA MiniPrep (ZYMO RESEARCH, #R2052) followed by DNAseI treatment according to the manufacturer's protocols. To check the quality of RNA isolated from plant tissues, electrophoresis in 1.2% agarose gels (conditions as described for DNA) can be performed.
4. Each of 20-μl reverse transcription reaction contained 5 μg of total RNA, 2 pmol of specific antisense primer, 1 mM dNTPs mix, 10 mM DTT, and 1 μl of PowerScript™ Reverse Transcriptase (BD Biosciences Clontech) in the buffer supplied by the manufacturer. The RNA and primers were preheated to 70 °C for 10 min and snap-cooled in ice water before adding the remaining components. The RT reactions were carried out for 1 h at 42 °C and were terminated by heating to 70 °C for 15 min. Then 1-μl aliquots of the reaction mixtures were used for PCR, with specific primer pairs designed for the selected cDNAs.
5. Use other donor vectors (for example, pH7CWG2, or pSITE-1NB, pSITE-2NA, pSITE-2NB, pSITE-4NA, or pSITE-4NB [9] which can be ordered on line from The Arabidopsis Information Resource (TAIR) web page (www.arabidopsis.org)) to create other stable and unstable fluorescent proteins.
6. Preparation of electrocompetent bacteria may be performed according to Dower et al. [10]. The same protocol can be used for *E. coli* and *A. tumefaciens.*
7. Use a toothpick and the Vaseline to draw the window along the edges of cover glass to prevent water evaporation and tissue crushing.

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