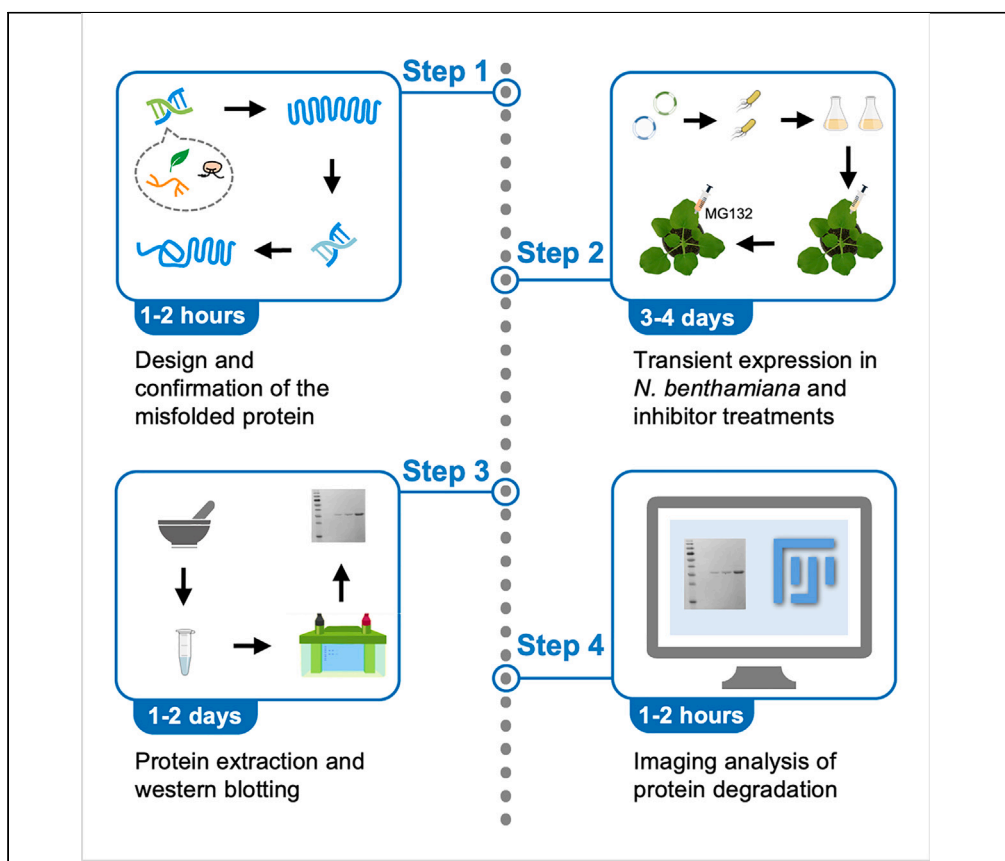


Protocol

Protocol for quantitative evaluation of misfolded protein degradation using *Agrobacterium*-mediated expression system in *Nicotiana benthamiana*



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Highlights
An *in planta* approach for quantitative evaluation of misfolded protein degradation

Instructions for designing misfolded proteins

Steps to explore roles of plant and non-plant proteins in misfolded protein degradation

Cellular protein homeostasis is maintained by the disposal of aggregated misfolded proteins. Here, we present a protocol for investigating the involvement of the proteins of interest in misfolded protein degradation via *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana*. We describe in detail the steps of misfolded protein design, transient protein expression in *N. benthamiana*, subsequent total protein extraction, and quantification of misfolded proteins through western blotting. This generalizable system can be used for misfolded proteins derived from various plants or microbes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Protocol for quantitative evaluation of misfolded protein degradation using *Agrobacterium*-mediated expression system in *Nicotiana benthamiana*Yang Cheng,^{1,3} Rui Meng,¹ Siqi Niu,¹ Hao Peng,² and Maofeng Jing^{1,3,4,*}¹Department of Plant Pathology, Key Laboratory of Soybean Disease and Pest Control (Ministry of Agriculture and Rural Affairs), Nanjing Agricultural University, Nanjing 210095, China²USDA-ARS, San Joaquin Valley Agricultural Sciences Center, Parlier, CA 93648, USA³Technical contact⁴Lead contact*Correspondence: jingmf@njau.edu.cn
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SUMMARY

Cellular protein homeostasis is maintained by the disposal of aggregated misfolded proteins. Here, we present a protocol for investigating the involvement of the proteins of interest in misfolded protein degradation via *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana*. We describe in detail the steps of misfolded protein design, transient protein expression in *N. benthamiana*, subsequent total protein extraction, and quantification of misfolded proteins through western blotting. This generalizable system can be used for misfolded proteins derived from various plants or microbes.

For complete details on the use and execution of this protocol, please refer to Ai et al.¹

BEFORE YOU BEGIN

Protein folding is a fundamental process to make proteins biologically active in eukaryotic cells. Abiotic and/or biotic stresses often disturb cellular protein homeostasis by causing the accumulation of misfolded/unfolded proteins.^{2,3} Eukaryotes have evolved a sensitive protein quality control (PQC) system to remove these abnormal proteins, thereby minimizing their aggregation and toxicity.^{2,4} PQC adopts various proteolytic apparatuses, with the ubiquitin (Ub)-proteasome system (UPS) degrading the majority of misfolded/unfolded proteins.⁵ To date, it's still technically challenging to investigate misfolded protein degradation in plants and microbes lacking efficient genetic transformation systems.

The experimental *Solanaceae* species *Nicotiana benthamiana* has become a plant model for studying both heterologous and endogenous proteins through *Agrobacterium*-mediated transient expression.^{6,7} This system is rapid, easy to set up, does not require tissue culture, and efficient for investigating proteins derived from different species. Moreover, *N. benthamiana* has a short life cycle and can rapidly produce recombinant proteins at high levels.⁶ Here, we utilize transient expression in *N. benthamiana* to explore and visualize the ability of heterologous or endogenous factors in degrading misfolded proteins *in vivo*.

Preparation of the plant material: *Nicotiana benthamiana*

© Timing: 4–5 weeks



Start preparing the plant material four to five weeks in advance as follows.

1. Mix the substrate for seedling cultivation and the vermiculite at a ratio of 3:1 and water the soil mixture fully. Then, sow *N. benthamiana* seeds in the soil.

Note: The substrate for seedling cultivation is the soil with good air permeability, water retention, and essential nutrients. This substrate can be replaced with loose, fertile, and well-drained sandy loam or yellow soil.

2. Incubate *N. benthamiana* seeds and the soil in plant growth chambers at an ambient temperature of 23°C under a 16-h light/8-h dark photoperiod for seven days.
3. Transfer the emerged seedlings individually to plastic pots filled with the same soil mixture. Water the pots with fertilizer.
4. Water the *N. benthamiana* plants on time to maintain soil humidity for normal growth. Apply the fertilizer once per week.
5. After 2–3 weeks, spread out *N. benthamiana* pots to avoid stunting.
6. Four- to five-week-old plants are ideal for transient expression and subsequent protein extraction.

△ **CRITICAL:** To achieve high protein accumulation, *N. benthamiana* plants should be cultivated under optimal growth conditions, watered carefully, and fertilized regularly. Sufficient spacing of 4–6 cm between each pot after 2–3 weeks is beneficial to the growth of *N. benthamiana*.

Preparation of plasmid constructs for transient expression in *N. benthamiana*

⌚ **Timing:** 1–2 h

Prepare plasmid constructs inserted with genes of interest for plant expression. Examples used here are the oomycete gene *PsBAG1* cloned from *Phytophthora sojae* strain P6497, the green fluorescent protein (GFP) and misfolded GFP (i.e., ΔGFP) genes, as described in Ai et al.¹

7. Insert *PsBAG1* coding sequence (CDS) into the pSuper-RFP vector.
8. Clone *GFP* and *ΔGFP* genes individually into the pBin-HA vector.

Note: Plant expression vectors harboring a CaMV 35S promoter, such as pSuper-RFP, pBin-HA, pBin-GFP2, and pCAMBIA-HA/FLAG, can be used for transient expression in *N. benthamiana*.

Note: pSuper-RFP and pBin-HA have red fluorescent protein (RFP) and HA tags fused at the C-terminal, respectively. The stop codons must be removed when designing the reverse primers.

Note: If the protein of interest has a functional domain at its C-terminal, consider tagging it at the N-terminal instead.

Design misfolded proteins

⌚ **Timing:** 1–2 h

Design misfolded proteins by mimicking their failure in folding properly under various stresses. Here, we describe the design of ΔGFP as an example.

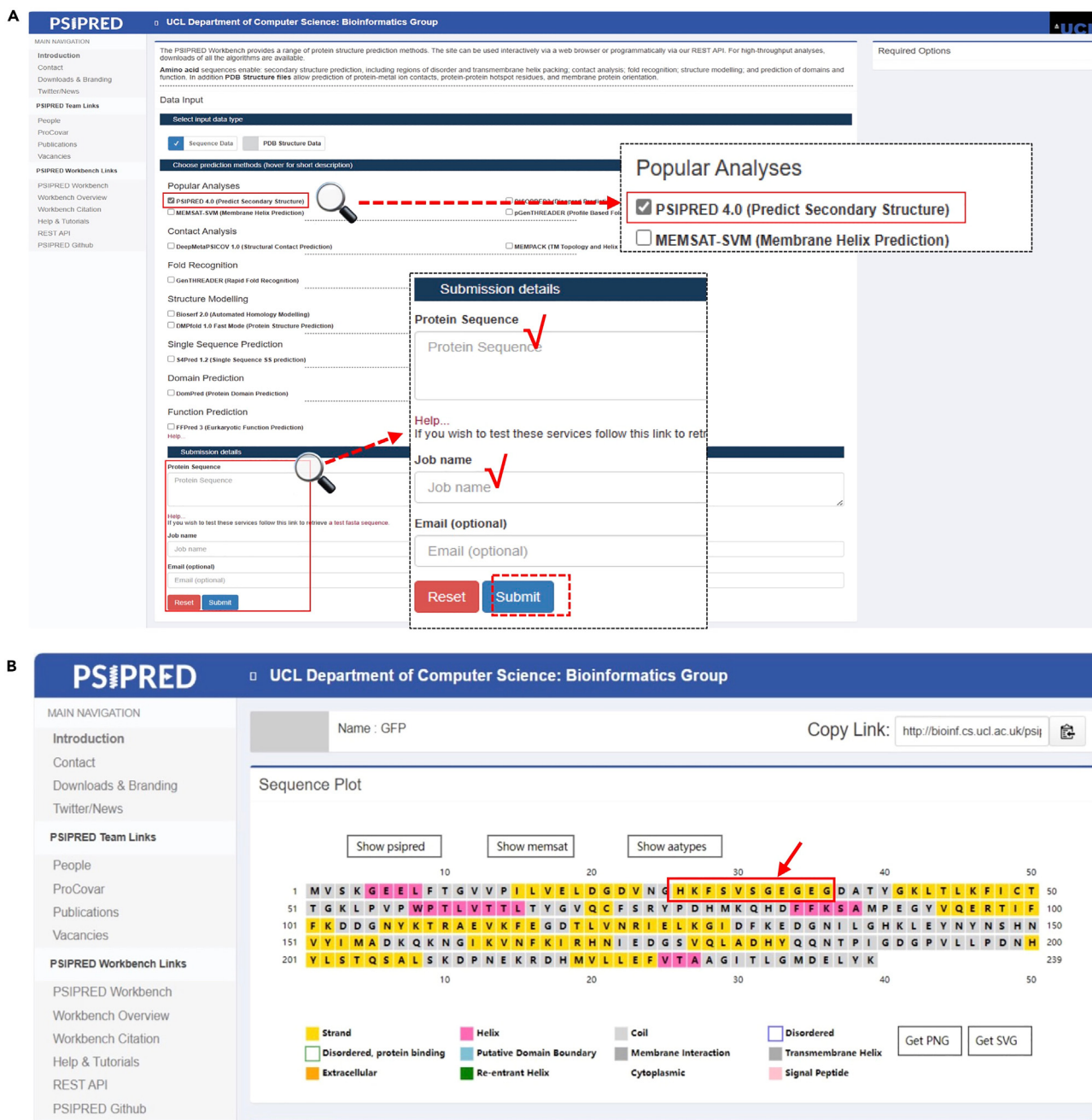


Figure 1. Predict secondary structure of the protein of interest using PSIPRED 4.0

(A) Screenshots of a step-by-step pipeline for protein secondary structure prediction using PSIPRED 4.0.

(B) The sequence plot shows the secondary structure of GFP. The second β -sheet is indicated by the red arrow.

9. Predict the secondary structure of a protein of interest.
 - a. Retrieve the GFP protein sequence.
 - b. Use the PSIPRED Workbench (<http://bioinf.cs.ucl.ac.uk/psipred/>) for protein structure prediction.
 - c. Choose the prediction method of "PSIPRED 4.0 (Predict Secondary Structure)" under the "Popular Analyses" section (Figure 1A).

- d. Paste the GFP protein sequence to the "Protein Sequence" box, and then fill out the job name and your email (Figure 1A).
- e. Click "Submit" to get the sequence plot and save it (Figure 1B).
- f. According to the sequence plot, delete the DNA sequence encoding the second β sheet from GFP CDS. Mark the misfolded GFP protein generated as Δ GFP (Figure 1B).

Note: Misfolded design for other proteins of interest or their interacting target proteins can be carried out using the same pipeline described above.

Note: Protein secondary structure can be predicted using other software such as SOPMA (https://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html).

10. Protein 3D structure prediction.

- a. Protein 3D structures can be predicted using ColabFold/AlphaFold2/MMseqs2 (<https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb#scrollTo=kObIAo-xetgx>) according to the instructions (Figures 2A–2C).

Note: This is an easy and convenient method to predict protein 3D structures using a public server.

Note: The default Settings of these programs work well generally. In some cases, extra model cycles are needed to improve accuracy.

Note: The proteomes of some model organisms, such as mouse, zebrafish, Arabidopsis, rice, etc., have been predicted by Alphafold2. Proteins of interest in these model species can be queried at <https://alphafold.ebi.ac.uk/>.

Alternatives: SWISS-MODEL (<https://swissmodel.expasy.org/interactive>) is a fully automated protein structure homology-modelling server that can also be used to predict 3D-structured models.

- b. Download prediction results and select the highest-ranked model for visualization in PyMOL.

11. Align the structural models of GFP and Δ GFP using PyMOL:

- a. Download and install PyMOL from <https://pymol.org/2/>, according to the instructions.
- b. Use PyMOL to open the Protein Data Bank (PDB) files obtained at step 10.b (Figure 3A).
- c. In the "all" section on the right, select GFP and Δ GFP protein files for alignment.

Note: Protein model colors can be changed in PyMOL by clicking on the "C" menu in the "all" section on the right and then selecting the color.

- d. In the "all" section on the right, click sequentially on the "A" menu in misfolded GFP, "align", "to molecule (*CA)", and select GFP (Figures 3B and 3C).
- e. Use mouse to rotate the model and detect the conformational changes in Δ GFP.
- f. In the top toolbar, click sequentially on "Display", "Sequence", and select the sequence with the conformational changes in misfolded GFP (Figure 3D).
- g. In the "all" section on the right, click on the "C" menu in the panel and select a different color to mark the misfolded structure (Figure 3E).

Optional: In the top toolbar, click sequentially on "Display", "Background", and select "white" or another color of interest to change the background.

- h. Save the PyMOL session or the image as needed (Figure 3F).

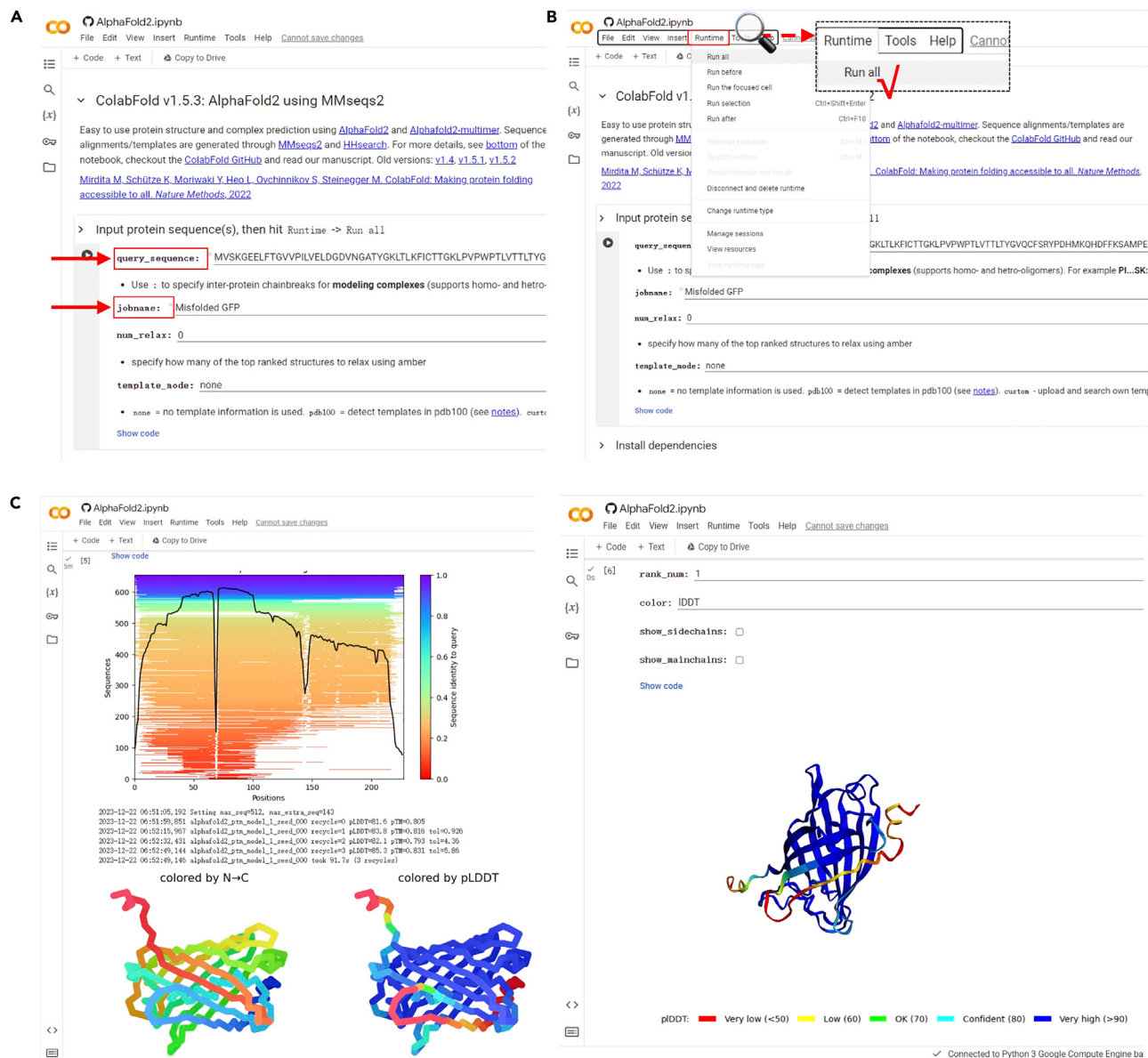


Figure 2. Predict 3D structural model of the protein of interest using AlphaFold2

(A and B) Submit the protein sequence and name to “query_sequence” and “jobname”, respectively. Click on “Runtime” and then on “Run all” to predict the 3D protein structure.

(C) Screenshots of the GFP 3D structural model predicted by AlphaFold2.ipynb.

⚠ **CRITICAL:** To design misfolded proteins, it is generally the best practice to delete the β -sheet, a key component of the 11-stranded β barrel that contributes to the stability of the GFP protein.^{8,9}

Detailed procedure for plasmid construction

⌚ **Timing:** 7–10 days

In this section, we describe how to construct the plasmid vectors used for *Agrobacterium*-mediated transformation and *in planta* expression.

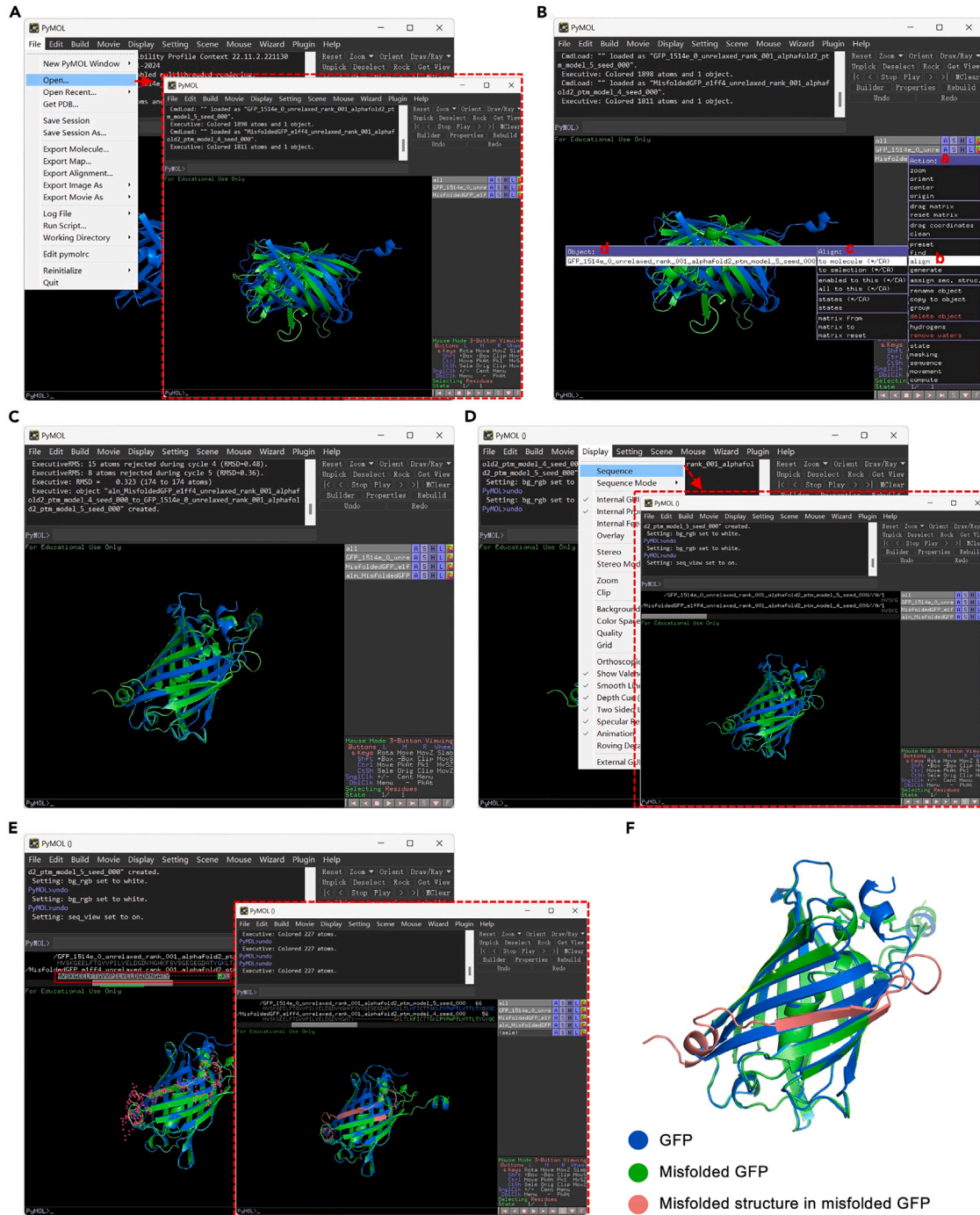


Figure 3. Align 3D structural models of selected proteins using PyMOL

- (A) Open the PDB files of GFP and misfolded GFP (Δ GFP).
- (B) The screenshot shows a step-by-step pipeline for aligning protein 3D structural models using PyMOL (a to d).
- (C) The screenshot shows the alignment result from step (B).
- (D) Click "Display" and then "Sequence" to display protein sequences.
- (E) The misfolded region is marked.
- (F) The 3D structural models of GFP and misfolded GFP are aligned.

12. Clone the gene of interest: using the ΔGFP gene as an example.
 - a. Design PCR primers for gene amplification.

Note: In the following steps, we will utilize homologous recombination to construct the recombinant construct. Therefore, when designing primers, PCR primers used for amplifying your genes must have 15–20 nucleotide overhangs on their 5' ends to ensure homology with the adjacent fragments of the linearized vector. Additionally, all 15–20 nucleotides providing the requisite homology with the linearized vector must be on the 5' end of the primer.

Note: To remove the second β -sheet of GFP, DNA fragments encoding the amino acids before and after the β -sheet can be cloned separately and then recombined into ΔGFP . Alternatively, the ΔGFP gene can be synthesized directly.

- b. Prepare the PCR reaction mix containing designed primers.
- c. Run standard PCR amplification (30–35 cycles) using the cycling conditions below:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	15 s	30–35 cycles
Annealing	58°C	15 s	
Extension	72°C	1 min	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

- d. Visualize the PCR products by 1% agarose gel electrophoresis.
- e. Purify PCR products with the FastPure Gel DNA Extraction Mini Kit (Vazyme), according to the manufacturer's instructions (<https://www.vazyme.com/product/18.html>).

Note: Single-band PCR product with the expected size can be directly purified using the FastPure Gel DNA Extraction Mini Kit. For PCR product with multiple bands including the correct-sized DNA fragment, the targeted band should be separated by gel cutting and then purified using the same kit.

13. Transform *Escherichia coli* strain DH5 α with the recombinant plant expression construct.
 - a. Digest the pSuper-RFP plasmid with XbaI and Sall-HF at 37°C for 30 min.
 - b. Visualize the product by 1% agarose gel electrophoresis.
 - c. Purify the linearized plasmid with FastPure Gel DNA Extraction Mini Kit, according to manufacturer's instructions (<https://www.vazyme.com/product/18.html>).
 - d. Perform a homologous recombination reaction between the PCR product obtained from step 12.e and linearized pSuper-RFP using the ClonExpress II One Step Cloning Kit (Vazyme). Incubate the reaction mix at 37°C for 30 min to generate the recombinant construct.
 - e. Transform *E. coli* cells by incubating the competent cells with the reaction mix on ice for 30 min, heat shocking at 42°C for 60–90 s, and then chilling on ice for 3–5 min.

Note: Water bath at 42°C and LB agar plates with the appropriate antibiotics should be ready before starting. Competed construct assembly reactions should be incubated on ice before transformation.

- f. Add 600 μ L LB to the Eppendorf tube. Shake at 37°C and 200 rpm for 1.5–2 h.
- g. Harvest the bacteria by centrifugation at 5000 \times g for 3–5 min. Resuspend the pellet in 100 μ L of LB.

h. Spread the bacterial suspension on LB agar plates containing 50 µg/mL kanamycin. Incubate the plates at 37°C for 12–16 h to let the transformed colonies grow.

Note: The pSuper-RFP, pBin-HA, and pCAMBIA-HA/FLAG enables transformed *E. coli* to grow on LB medium containing kanamycin.

14. Identification of *E. coli* transformants harboring the recombinant constructs by colony PCR.
 - a. Prepare a PCR master mix including forward (e.g., pSuper-Fw) and reverse (e.g., pSuper-Rv) primers targeting the vector (e.g., pSuper-RFP) in a 1.5 mL Eppendorf tube. Dispense 15-µL aliquots into sterile PCR tubes.

Note: Submit the linearized vector sequence required for primer design (approximately 500–800 bp on both sides of the restriction enzyme site) to Primer 3 Plus (<https://www.primer3plus.com/index.html>). Set “Task” as “pick primer list,” click “Pick primers” to generate the primers list, and then select the Forward (Fw) and Reverse (Rv) primers from both ends of the restriction enzyme site to ensure the detection product size falls within the range of 300–600 bp.

PCR reaction master mix	
Reagent	Amount
2 × Rapid Taq Master Mix	7.5 µL
pSuper-Fw (10 nM)	0.2 µL
pSuper-Rv (10 nM)	0.2 µL
ddH ₂ O	7.1 µL
Total	15 µL

Note: The primer pair pSuper-Fw and pSuper-Rv are used for pSuper-RFP recombinant clone identification.

- b. Screen white colonies selected in step 13.h.
- c. Pick individual colonies from the plate with sterile P10 micropipette tips and inoculate to a new LB agar plate containing 50 µg/mL kanamycin. Then soak individual tips in the PCR mix tubes for 5–30 s. A total of eight independent colonies were selected for each construct.

Alternatives: In the process of identifying recombinant constructs, restriction enzyme mapping can be used as a screening method. Or directly screen white colonies selected in step 13.h and send them to the company for sequencing to get the correct plasmid.

Note: PCR tube should be marked to their corresponding colonies on the new LB agar plate.

- d. Incubate the new plates at 37°C for 12–16 h.
- e. Run standard PCR amplifications of 30–35 cycles on a thermal cycler using the following cycling conditions:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	15 s	30–35 cycles
Annealing	58°C	15 s	
Extension	72°C	20 s	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

- f. Resolve 10 μ L of each PCR reaction by 1% agarose gel electrophoresis.
 - g. Mark positive transformants identified from the new LB agar plate in step 14.c and store at 4°C.
15. Plasmid propagation and extraction from transformed *E. coli* cells.
- a. Pick three positive colonies to individually inoculate LB liquid medium containing 50 μ g/mL kanamycin. Incubate the cultures at 37°C and shake at 200 rpm for 12–16 h.
 - b. Prepare the plasmids using the FastPure Plasmid Mini Kit (Vazyme).
 - c. Validate the recombinant plant expression vectors by using the primers pSuper-Fw and pSuper-Rv to amplify sequence for Sanger sequencing.
 - d. Dissolve the correct plasmids in Milli-Q water or the FastPure Plasmid Mini Kit elution buffer. Store at –20°C for further use.

Alternatives: Other commercial Gel Extraction Kit, PCR reagents, Exnase II used for constructing recombinant vectors, and Plasmid Extraction Kit can be used for the same purposes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GFP-Tag(7G9) mouse antibody (1:1,000)	Abmart	Cat#M20004; RRID: AB_2619674
Goat anti-mouse IgG HRP (1:10,000)	Abmart	Cat#M21001; RRID: AB_2713950
RFP-tag antibody (1:5,000)	Abmart	Cat#MA9041; RRID: AB_2846170
Actin (26F7) mAb for PLANTs (1:5,000)	Abmart	Cat#M20009; RRID: AB_2936239
Bacterial and virus strains		
<i>Escherichia coli</i> strain DH5a	WEIDI (Shanghai)	CAT#DL1001
<i>Agrobacterium tumefaciens</i> strain GV3101	WEIDI (Shanghai)	Cat#AE1001
Chemicals, peptides, and recombinant proteins		
Agar	Solarbio	Cat#A8190
Yeast extract	Thermo Fisher Scientific	Cat#LP0021B
Peptone	Sangon	Cat#A505247
Kanamycin	Solarbio	Cat#K8020
Rifampicin	Solarbio	Cat#IR0110
Magnesium chloride (MgCl ₂)	Solarbio	Cat#M8161
MG132	Beyotime	Cat#S1748
HEPES	Sigma-Aldrich	Cat#H3375
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P9541
Ethylenediaminetetraacetic acid (EDTA)	Solarbio	Cat#E8040
Protease inhibitor cocktail	Warbio	Cat#PIC02
Potassium hydroxide (KOH)	Sigma-Aldrich	Cat#P5958
DL-dithiothreitol (DTT)	Sigma-Aldrich	Cat#D9779
Acryl/bis 30% solution (29:1)	Sangon	Cat#B546017
Tris-HCl, pH6.8 (1 M)	Beyotime	Cat#ST768
Tris-HCl, pH8.8 (1.5 M)	Beyotime	Cat#ST789
Ammonium persulfate (APS)	https://www.reagent.cn/	Cat#10002618
Sodium dodecyl sulfate (SDS)	Solarbio	Cat#S8010
N,N,N,N-tetramethylethylenediamine (TEMED)	Sigma-Aldrich	Cat#T9281
Tris-Gly, powder	Beyotime	Cat#P0014A-50bgs
Methanol	https://www.reagent.cn/	Cat#10014118
Skim milk powder	Solarbio	Cat#LP0033B
Tris (hydroxymethyl) aminomethane (Tris-base)	Solarbio	Cat#T8060
Sodium chloride (NaCl)	Sangon	Cat#A501218
Triton X-100	Solarbio	Cat#T8200
Tween 20	Solarbio	Cat#T8220

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
5× SDS-loading buffer	Zomanbio	Cat#ZS306
Liquid nitrogen	N/A	N/A
Critical commercial assays		
Phanta Max super-fidelity DNA polymerase	Vazyme	Cat#P505-d1
ClonExpress II one step cloning kit	Vazyme	Cat#C112-02
2 × Rapid Taq Master Mix	Vazyme	Cat#P222-01
FastPure gel DNA extraction mini kit	Vazyme	Cat#DC301-01
FastPure plasmid mini kit	Vazyme	Cat#DC201-01
180 kDa prestained protein marker	Vazyme	Cat#MP102-02
XbaI	NEB	Cat#R0145S
Sall-HF	NEB	Cat#R3138V
1× Tris/glycine/SDS buffer	Beyotime	Cat#P0014A-50bgs
Enhanced chemiluminescence (ECL) substrate	Beierbo	Cat#BB-21001
Deposited data		
Original western blot images	This protocol	https://data.mendeley.com/preview/snbg5tywfy
Experimental models: Organisms/strains		
<i>Nicotiana benthamiana</i> : LAB wild-type	Ai et al. ¹	N/A
<i>Phytophthora sojae</i> P6497	Ai et al. ¹	N/A
Oligonucleotides		
Primer for amplification of PsBAG1, F1: CACCAAATCGACTCTAGAAT GGAGGCCAGACAAC	Ai et al. ¹	N/A
Primer for amplification of PsBAG1, R1: TCGGAGGAGGCCATGTGAC CCAGACGAAGCAGTCCTGCG	Ai et al. ¹	N/A
Primer for amplification of ΔGFP, F1: CGATAGGGTACCCCGGGAT GGTGAGCAAGGGCGAG	Ai et al. ¹	N/A
Primer for amplification of ΔGFP, R1: CTTGCCGTAGGTGGCGC CGTTTACGTCGCC	Ai et al. ¹	N/A
Primer for amplification of ΔGFP, F2: GGCGACGTAAACGGCGC CACCTACGGCAAG	Ai et al. ¹	N/A
Primer for amplification of ΔGFP, R2: GGATCCGTCGACCCCGGGCT TGACAGCTCGTCCAT	Ai et al. ¹	N/A
pSuper-Fw: ATCTTCCCAAATTACCAATAC	Ai et al. ¹	N/A
pSuper-Rv: TGGCCGTTACGGAGCCC	Ai et al. ¹	N/A
Recombinant DNA		
Plasmid: pBin-HA	Ai et al. ¹	N/A
Plasmid: pSuper-RFP	Ai et al. ¹	N/A
Plasmid: pBin-GFP2	BioVector	http://www.biovector.net/product/425587.html
Plasmid: pCAMBIA-HA/FLAG	Abcam	Cat# ab275754
Software and algorithms		
ImageJ	Software	https://imagej.nih.gov/ij/
PyMOL	Software	https://pymol.org/2/
Other		
PVDF membrane	Merck Millipore	Cat#ISEQ00010
Centrifuge 5418 R	Eppendorf	Cat#Centrifuge 5418 R
MicroPulser electroporator	Bio-Rad	Cat#1652100
eBlot L1	GenScript	Cat#L00686C
Tanon-6600	Tanon	Cat#Tanon 6600
PowerPac basic power supply	Bio-Rad	Cat#1645050
Mini-PROTEAN tetra vertical electrophoresis cell	Bio-Rad	Cat#1658004

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<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
1.5 mL Eppendorf tube	Eppendorf	Cat#0030125150
2.0 mL Eppendorf tube	Eppendorf	Cat#0030120094
Substrate for seedling cultivation	This protocol	http://www.ding-ye.cn/ggscymjz/2513.html
Fertilizer	This protocol	http://www.ynhzny.com/index.php?_m=mod_product&a=view&p_id=318

MATERIALS AND EQUIPMENT

Preparation of stock solutions

⌚ Timing: 1–2 days

This section describes the preparation of concentrated stock solutions used in this protocol.

- **Kanamycin sulfate (Kan), 50 mg/mL:** Dissolve 2.5 g of Kan in 45 mL of ddH₂O, bring the final volume to 50 mL, and sterilize by filtration with a 0.2 μm filter. Store in 1-mL aliquots at –20°C and use within 6 months.
- **Rifampicin (Rif), 50 mg/mL:** Dissolve 2.5 mg of Rif in 45 mL of DMSO, bring the final volume to 50 mL, and sterilize by filtration with a 0.2 μm filter. Store in 1-mL aliquots at –20°C in the dark and use within 4–6 months.
- **Dithiothreitol (DTT), 1 M:** Dissolve 1.54 g of DTT in 10 mL of ddH₂O. Store in 1-mL aliquots at –20°C and use within 4–6 months.
- **Sodium dodecyl sulfate (SDS), 10% (w/v):** Dissolve 5 g of SDS in 45 mL of ddH₂O and bring the final volume to 50 mL. Store at 23°C–25°C and use within 4–6 months.
- **Ammonium persulfate (APS), 10% (w/v):** Dissolve 5 g of APS in 45 mL of ddH₂O and bring the final volume to 50 mL. Store at 23°C–25°C and use within 4–6 months.
- **Magnesium chloride (MgCl₂), 1 M:** Dissolve 23.8025 g of MgCl₂ in 250 mL (final volume) of ddH₂O. Store at 23°C–25°C and use within 1 year.
- **MG132, 10 μM:** Dissolve 5 mg of MG132 in 1 mL of DMSO. Store at –20°C and use within 4–6 months.

Preparation of media

⌚ Timing: 1 day

LB liquid medium		
Reagent	Final concentration	Amount
NaCl	1% (w/v)	10 g
Yeast extract	2% (w/v)	5 g
Peptone	1% (w/v)	10 g
ddH ₂ O	N/A	Bring the final volume to 1 L
Total	N/A	1 L

Note: Pour 200 mL of medium into each 250-mL Erlenmeyer flask. Autoclave at 121°C for 20 min. Store at 23°C–25°C and use within 1–2 months.

LB agar medium		
Reagent	Final concentration	Amount
NaCl	1% (w/v)	10 g
Yeast extract	2% (w/v)	5 g
Peptone	1% (w/v)	10 g
Agar	1.5% (w/v)	15 g
ddH ₂ O	N/A	Bring the final volume to 1 L
Total	N/A	1 L

Note: Pour 200 mL medium into each Erlenmeyer flask (250 mL). Autoclave at 121°C for 20 min and store at 23°C–25°C for 1–2 months.

Alternatives: Bacterial peptone can be replaced by bacterial tryptone in the above media.

Preparation of buffers

⌚ Timing: 1–2 days

Infiltration buffer		
Reagent	Final concentration	Amount
MgCl ₂ (1 M)	10 mM	5 mL
ddH ₂ O	N/A	45 mL
Total	N/A	50 mL

Note: Prepare fresh infiltration buffer immediately before use at 23°C–25°C.

HEPES buffer		
Reagent	Final concentration	Amount
HEPES	50 mM	5.96 g
KCl	150 mM	5.59 g
EDTA	1 mM	0.14615 g
ddH ₂ O	N/A	Bring the final volume to 500 mL
Total	N/A	500 mL

Note: Adjust the pH to 7.5 with KOH. Store at 4°C and use within 4–6 months.

Protein extraction buffer		
Reagent	Final concentration	Amount
Triton X-100	0.5% (v/v)	1 mL
DTT (1 M)	1 μM	20 μL
Protease inhibitor cocktail	N/A	20 μL
HEPES buffer	N/A	Bring the final volume to 20 mL
Total	N/A	20 mL

Note: The buffer should be prepared fresh each time right before protein extraction. DTT and Protease inhibitor cocktail should be stored at –20°C.

SDS-PAGE: 12% resolving gel

Reagent	Final concentration	Amount
Acryl/Bis 30% Solution (29:1)	40% (v/v)	2 mL
Tris-HCl, pH8.8 (1.5 M)	0.39 M	1.3 mL
SDS (10%)	0.1% (v/v)	50 μ L
APS (10%)	0.1% (v/v)	50 μ L
TEMED	N/A	2 μ L
ddH ₂ O	N/A	Bring the final volume to 5 mL
Total	N/A	5 mL

SDS-PAGE: stacking gel

Reagent	Final concentration	Amount
Acryl/Bis 30% Solution (29:1)	16.5% (v/v)	0.33 mL
Tris-HCl, pH6.8 (1 M)	0.125 M	0.25 mL
SDS (10%)	0.1% (v/v)	20 μ L
APS (10%)	0.1% (v/v)	20 μ L
TEMED	N/A	2 μ L
ddH ₂ O	N/A	Bring the final volume to 2 mL
Total	N/A	2 mL

Note: We recommend preparing fresh SDS-PAGE each time before use. The SDS-PAGE can be stored at 4°C for up to 2–3 days before use.

△ CRITICAL: When preparing the resolving gel and the stacking gel, the solution need to be well mixed and solidified.

10x TBS buffer (pH = 7.6).

Reagent	Final concentration	Amount
Tris-base	2.42% (w/v)	24.2 g
NaCl	8% (w/v)	80 g
ddH ₂ O	N/A	Bring the final volume to 1 L
Total	N/A	1 L

Note: Adjust the pH to 7.6 with HCl. The 10x TBS buffer can be stored at 20°C–25°C for up to 1 year.

TBST buffer: add 1 mL of Triton X-100 in 1 L of 1x TBS buffer.

Note: Prepare fresh buffer each time before use. The TBST buffer can be stored at 20°C–25°C.

STEP-BY-STEP METHOD DETAILS

In this protocol, we outline the steps for the infiltration of *Agrobacterium tumefaciens* (step 1 to 3), treatment with protein inhibitors (steps 4 to 7), extraction of plant proteins (steps 8 to 18), and detection and quantification of proteins (steps 19 to 31).

Preparation of *Agrobacterium tumefaciens* for infiltration

⌚ Timing: 2 h

In this section, the plant expression vectors are transformed into *Agrobacterium tumefaciens* strain GV3101, and expressed in *N. benthamiana* leaves by infiltration with appropriate transformation buffer.

1. Transformation of *Agrobacterium* cells.
 - a. Thaw on ice the aliquots of electro-competent *Agrobacterium tumefaciens* (strain GV3101) cells.
 - b. Mix a 100- μ L cell aliquot with 100 ng of plant expression vector in a 1.5-mL Eppendorf tube.
 - c. Pipette the mix into a prechilled 0.2-cm electroporation cuvette.
 - d. Pulse the cells at 2.5 kV with a MicroPulser Electroporator.
 - e. Add 500–800 μ L of LB media to the cuvette, mix well, and immediately transfer the mixture into a clean 1.5-mL Eppendorf tube.
 - f. Shake the cells at 28°C and 220 rpm for 1.5–2 h.
 - g. Spread 200- μ L bacterial suspension on each LB agar plates supplemented with 50 μ g/mL kanamycin and 50 μ g/mL rifampicin. Incubate the plates at 28°C for 48–72 h.

Optional: Confirm the presence of desired constructs in *Agrobacterium* transformants by colony PCR, as described at step 14 in [detailed procedure for plasmid construction](#).

2. Pick single colonies harboring the desired plant expression vectors and inoculate each colony to 3–4 mL of liquid LB with 50 μ g/mL kanamycin and 50 μ g/mL rifampicin in a 10-mL tube. Shake the tubes at 28°C and 220 rpm for 24–48 h.
3. Preparation of bacterial suspension for agroinfiltration.
 - a. Harvest the bacteria by centrifugation at 8000 \times g for 3–5 min. Discard the supernatant by pipetting.
 - b. Resuspend the pelleted cells in fresh 10 mM MgCl₂.
 - c. Repeat step 3.a and b twice.
 - d. Resuspend the pelleted cells in 10 mM MgCl₂ to an OD₆₀₀ of 0.4.
 - e. Incubate the suspension at 28°C in the dark for 1–2 h before infiltration.

Transient expression in *N. benthamiana* and protease inhibitor treatment

⌚ Timing: 48–60 h

This section describes the steps of the transient expression in *N. benthamiana*. The protein of interest is co-expressed with Δ GFP to explore its participation in degrading misfolded proteins (i.e., Δ GFP) by utilizing the protein quality control system in *N. benthamiana*.

4. Mix *A. tumefaciens* suspensions harboring the constructs expressing Δ GFP and the protein of interest at a 1:1 ratio before infiltration.

Note: 6–10 mL of *A. tumefaciens* culture is sufficient for infiltrating 3–5 *N. benthamiana* leaves, with about 2 mL suspension used per leaf on average.

5. Fully infiltrate the suspension mixture to abaxial leaf surface with a 1-mL needleless syringe. 3–5 leaves can be infiltrated per *N. benthamiana* plant ([Figures 4A and 4B](#)).

Note: Choose young and healthy leaves ([Figure 4C](#)) and water *N. benthamiana* plants one day earlier to facilitate the infiltration.

Note: When infiltrating, simply press the syringe on the underside of the leaf (avoiding the veins), and apply counter-pressure with your finger on the other side. Then, press the plunger of the syringe with your thumb to slowly infiltrate the suspension into the *N. benthamiana* leaf. Sufficient infiltration is indicated by translucence of the whole leaf area.



Figure 4. Transient protein expression in *Nicotiana benthamiana* leaves and confirmation of misfolded protein degradation by Western blotting

- (A) Select 4- to 5-week-old plants for agroinfiltration.
- (B) Infiltrate *N. benthamiana* leaves with *Agrobacterium tumefaciens* suspension using a 1-mL needleless syringe.
- (C) Translucent *N. benthamiana* leaves after agroinfiltration.
- (D) At 36 h post infiltration (hpi), infiltrate MG132 into half of the pre-labeled leaf with a 1-mL needleless syringe.
- (E) Collect infiltrated leaves at 48 hpi.
- (F) Remove the petiole and the middle vein from leaf base with scissors.
- (G) Weigh the leaves.
- (H) Add quartz sand and protein extraction buffer to the mortar.
- (I) Grind leaf samples on the ice.
- (J) Collect ground leaf suspension in a 2.0-mL Eppendorf tube.
- (K) Centrifuge the samples at 4°C and 12,000 × g 15 min.
- (L) Transfer the supernatant to a new 1.5-mL tube.

Figure 4. Continued

- (M) Mix 80 μ L of supernatant with 20 μ L of 5 \times SDS-loading Buffer.
- (N) Run SDS-PAGE using the Mini-PROTEAN Tetra Vertical Electrophoresis Cell.
- (O) Transfer the proteins to a PVDF membrane using eBlot L1.
- (P) After Western blotting, the membrane is visualized using an automatic image analysis system (Tanon-6600).

6. Infiltrate half of each leaf with the classic proteasome inhibitor MG132 (10 μ M) at 24 h post agroinfiltration. Infiltrate the other half with water as the mock (Figure 4D).
7. Keep infiltrated plants in the greenhouse at 22°C and long day conditions for 24–36 h.

Note: When infiltrating *A. tumefaciens* harboring different genes, avoid cross-contamination and damages to the leaves.

Protein extraction from plant tissues

⌚ Timing: 2 days

In this section, we describe the steps for extracting plant proteins.

8. At 48–60 h post agroinfiltration, cut the leaves off, remove the petioles and middle veins from the base with scissors (Figures 4E and 4F).
9. Collect and weigh the leaves. Then place them in a mortar and add a spoon of quartz sand (Figures 4G and 4H).
10. Add 300 μ L of protein extraction buffer supplemented with 0.3 μ L of protease inhibitor cocktail per 0.1 g of leaves (Figure 4H).
11. Grind the leaf sample thoroughly using a pestle (Figure 4I).

Note: The mortars and pestles should be precooled and placed on ice when grinding samples.

12. Transfer each ground leaf suspension to a 2.0-mL Eppendorf tube (Figure 4J).
13. Keep the samples on ice or at 4°C and mix for 5–15 min.
14. Centrifugate at 12,000 \times g and 4°C for 15 min (Figure 4K).
15. Discard the debris pellets. Transfer each supernatant to a pre-cooled 1.5-mL Eppendorf tube (Figure 4L).
16. Pipette 80 μ L of supernatant to a new 1.5-mL Eppendorf tube. Add 20 μ L of 5 \times SDS-loading Buffer (Zomanbio) and mix well according to the manufacturer's recommendation <http://zomanbio.com/ueditor/php/upload/20150615/14343489241178.pdf> (Figure 4M).
17. Incubate the tubes at 100°C for 8–10 min.
18. Place the tubes at 25°C for 2 min before carrying out the Western blot or store at –20°C for future use.

Note: Extractions should be performed on time (48–60 h after agroinfiltration) to get high quality proteins.

Note: Harvested infiltrated leaves can be stored temporarily at –80°C or in liquid nitrogen. However, the samples should be ground into a fine powder in liquid nitrogen using a mortar and pestle. Weigh the powder, add protein extraction buffer, and then perform steps 13–18.

Western blotting

⌚ Timing: 6–8 h

In this section, we outline the steps for detecting proteins of interest in the extracts by Western blot. Visualize the proteins using specific antibodies to confirm bait expression and its capacity to degrade misfolded proteins.

19. Load 10–20 μL of each sample from step 18 on an SDS-PAGE gel (Figure 4N).
20. Load 5 μL of the ladder Precision Protein 180 kDa Prestained Protein Marker (Vazyme) (Figure 4N).
21. Run the gel in an electrophoresis cell with 1 \times Tris/Glycine/SDS buffer (Figure 4N).
22. Transfer the proteins to a 0.2- μm PVDF membrane (Merck Millipore; pretreated with methanol for 15–20 s) in eBlot L1 (GenScript) for 15 min (Figure 4N).
23. Block the membrane with 10 mL of TBS buffer (pH 7.4) containing 3% non-fat dry milk. Shake at 25°C and 40 rpm for 1 h.
24. Recycle the blocking buffer to a 10 mL tube. Then wash the membrane 3 times with TBST (TBS with 0.1% Tween 20).
25. Add the appropriate antibody to TBST containing 3% non-fat dry milk. Incubate with the membrane at 25°C for 1.5 h.
 - a. Use an anti-GFP antibody diluted by 1:1,000 in the Blocking Buffer to determine the accumulation of GFP-tagged misfolded proteins *in planta*.

Note: When another gene replaces the ΔGFP gene and is cloned into the pBin-HA vector, anti-HA antibodies can be used to detect the content of proteins.

- b. Use an anti-RFP antibody diluted by 1:5,000 in the Blocking Buffer to check for the presence of RFP-tagged proteins.
 - c. Use an anti-actin antibody for plants diluted by 1:5,000 in the Blocking Buffer to check for the presence of plant actin proteins as loading controls.
26. Recycle the buffer in step 25 and wash the membrane 3 times with TBST.
27. Incubate the membrane with the goat anti-mouse IgG antibody at a ratio of 1:10,000 in TBST at 25°C for 45 min.
28. Recycle the buffer containing the secondary antibody. Wash the membrane 3 times with TBST.
29. Add the enhanced chemiluminescence (ECL) substrate of choice. Then visualize the membrane using Tanon-6600 (Figures 4O and 5C).

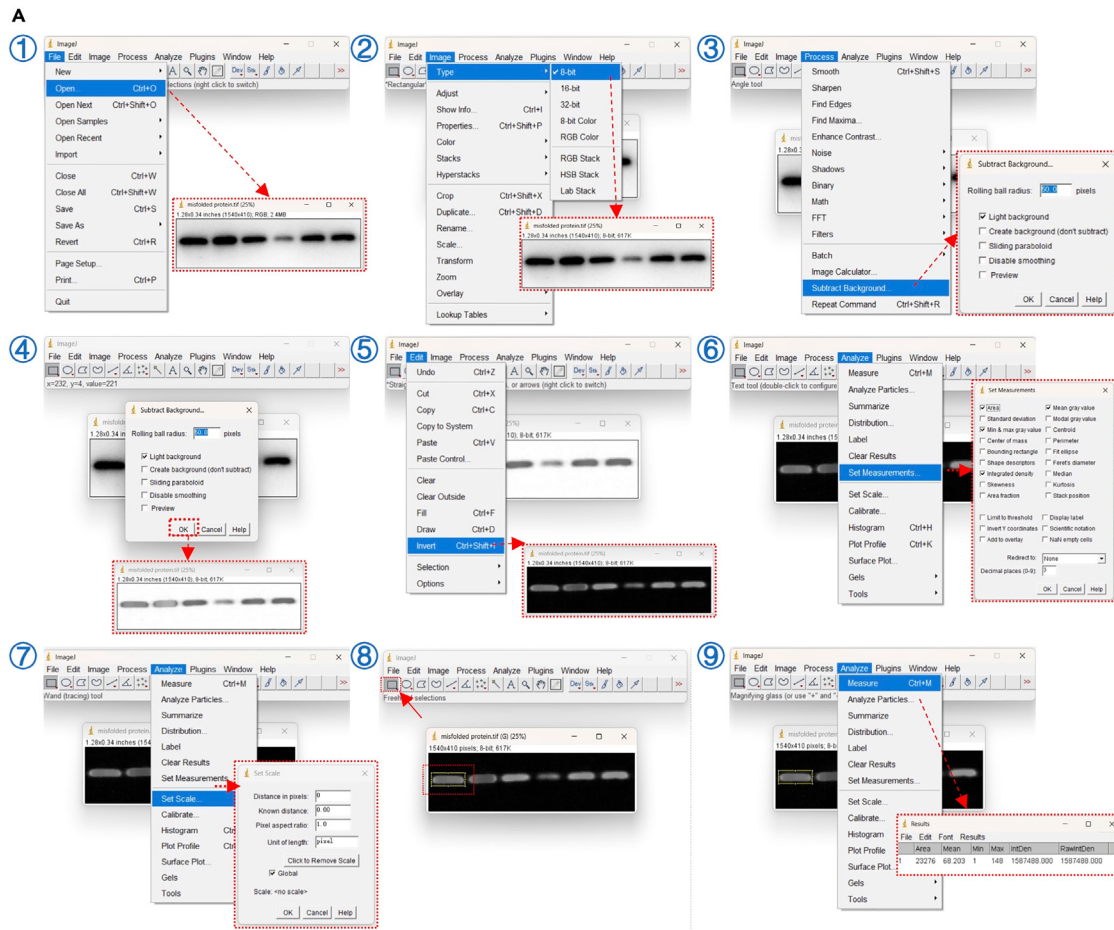
Note: The recycled buffer from steps 24, 26, and 28 can be stored at -20°C and used at least three more times.

Quantification of protein bands in western blots using ImageJ

⌚ Timing: 1 h

In this section, we describe steps for quantifying proteins using ImageJ. ImageJ is used to quantify and normalize the intensity values of protein bands based on their corresponding loading control bands.

30. Open the image of protein bands visualized by the antibodies in ImageJ and act as follows (see Figure 5A):
 - a. In the toolbar, click on the “Image” menu and then on “Type”. Select “8-bit”.
 - b. Click “Process” and then select “Subtract Background”. Keep the default parameter (50) to eliminate background effects.
 - c. Click “Edit” and then select “Invert” to transform the black protein bands to white ones.
 - d. Set up the measurement criteria and apply to all the blots to analyze.
 - i. In the toolbar, click on the “Analyze” menu and then select “Set Measurements”.
 - ii. In the checkboxes, keep “Area”, “Mean gray value”, and “Integrated density” checked.
 - e. Next, click on the “Analyze” menu in the toolbar to select “set scale”.



B

Samples	GFP (Gray values)	Actin (Gray values)	Normalization of gray values
GFP + RFP	1587488	8745	1.00
GFP + PsBAG1-RFP	1269194	6165	1.13
Δ GFP + RFP	1561002	7886	1.09
Δ GFP + PsBAG1-RFP	756248	13349	0.31
Δ GFP + RFP (MG132)	1509648	11501	0.72
Δ GFP + PsBAG1-RFP (MG132)	1568288	6222	1.39

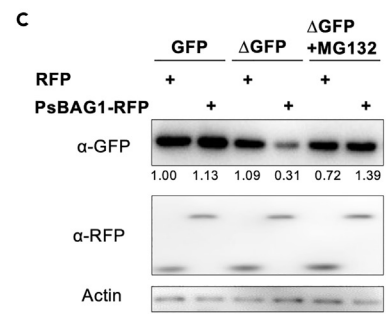


Figure 5. Quantification of misfolded protein degradation using ImageJ

(A) The screenshots show a step-by-step pipeline for quantifying protein band intensity using ImageJ.

(B) Calculate normalized signal intensity values of GFP-tagged protein bands based on their corresponding loading controls detected by the anti-actin antibody.

(C) Western blots showing differential accumulation of misfolded protein when co-expressed with the protein of interest. Numbers below blots indicate their relative intensities. The normal GFP control or misfolded GFP (Δ GFP) was co-expressed with either PsBAG1-RFP or the RFP control. PsBAG1-RFP, but not RFP, induced significant degradation of Δ GFP. PsBAG1-mediated Δ GFP degradation could be suppressed by MG132.

- i. Change inch to pixel in "unit of length" and keep "global" checked.
- f. Select the "Rectangle" tool and draw a frame around the largest band.
 - i. Adjust frame size to cover the minimum area.
 - ii. Click on the "Analyze" menu in the toolbar and then on "Measure" to get the intensity values.

- iii. Use the same frame for measuring the intensity values of all the protein bands in the image.
 - g. Next, export and save the values in “Results” into a spreadsheet. The “IntDen” in “Results” is the integrated gray value.
31. Quantification and normalization of the gray values of protein bands according to their corresponding loading control bands marked by the anti-actin antibody.
 - a. Measure the gray values of protein bands and their corresponding loading control bands marked by the anti-GFP and the anti-actin antibodies, respectively (Figure 5B).
 - b. Normalize the relative gray value of a band based on its corresponding loading control.
 - i. Calculate the relative gray values of all the samples.
 - ii. Finally, mark the relative value of the control group (GFP + RFP) as 1. Result of the treated group is the fold change relative to the control group (Figures 5B and 5C).

Note: To facilitate the quantification of proteins bands, save high-resolution images or scans from the blots or the films, respectively.

Note: The loading control bands can be also detected by using Ponceau S.

EXPECTED OUTCOMES

This protocol provides a convenient method that quantifies the participation of selected proteins in misfolded protein degradation. Here, Cheng and co-workers mimic the accumulation of misfolded GFP proteins by agroinfiltrating a Δ GFP-expressing construct into *N. benthamiana* leaves. Then, misfolded protein degradation induced by the protein of interest (e.g., PsBAG1) can be visualized and quantified by Western blot (Figure 5C). Compare to the normal GFP control, misfolded GFP can be degraded in the presence of PsBAG1. PsBAG1-induced Δ GFP degradation can be inhibited by the classic proteasome inhibitor MG132. This system is suitable for identifying plant proteins triggering misfolded protein degradation in response to stresses such as temperature variation, pathogen invasion, and pest infestation. In addition, this system can be used for exploring similar factors in microbes that are difficult to transform.

LIMITATIONS

Using *N. benthamiana* as a platform, this protocol works well in characterizing plant or microbial proteins responsible for degrading misfolded proteins. However, misfolded proteins aggregated in eukaryotic cells can be disposed through various pathways including UPS and lysosome-mediated autophagy.¹⁰ This protocol is limited to the exploration of UPS-mediated misfolded protein degradation.

TROUBLESHOOTING

Problem 1

Protein secondary structures predicted with PSIPRED 4.0 and another software are inconsistent (related to “[design misfolded proteins](#)” step).

Potential solution

Predict the β -sheets using different software or websites such as SOPMA and CFSSP. Delete the second β -sheet predicted by each software/website. Then, predict the 3D structures of different versions of the modified protein sequences after β -sheet deletion. Compare the prediction results to verify the misfolded structure and choose the best version to proceed experimentally.

Problem 2

After agroinfiltration, *N. benthamiana* plants show abnormal phenotypes such as leaf wilting (related to “[transient expression in *N. benthamiana* and protease inhibitor treatment](#)” step).

Potential solution

Keep the plants in a stable environment before, during, and after infiltration. Remove *A. tumefaciens* suspension from leaf surface with absorbent paper after infiltration. Water the plants on time.

Problem 3

The amounts of proteins transiently expressed in different plant samples are highly variable (related to “Western blotting” step).

Potential solution

Select *N. benthamiana* plants under identical growth conditions and leaves of similar sizes and positions for infiltration. Keep the OD₆₀₀ values of *A. tumefaciens* suspensions consistent.

Problem 4

Protein band intensities in the Western blot are too strong or too weak (related to “Western blotting” step).

Potential solution

Adjust the weight of *N. benthamiana* leaves used for protein extraction, the amounts of proteins loaded on SDS-PAGE, the blot exposure time, and/or the amount of chemiluminescence substrates. High-sensitivity chemiluminescence substrates can be used to generate stronger bands in Western blot detection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maofeng Jing (jingmf@njau.edu.cn).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contacts, Maofeng Jing (jingmf@njau.edu.cn) and Yang Cheng (2021202008@stu.njau.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets.

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AUTHOR CONTRIBUTIONS

Y.C. performed experiments and wrote the manuscript. R.M. and S.N. performed experiments. H.P. participated in the editing of the manuscript. M.J. designed the protocol strategy, conceived the project, supervised the study, and wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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