Chapter 6

Generation of Artificial N-end Rule Substrate Proteins In Vivo and In Vitro

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Abstract

In order to determine the stability of a protein or protein fragment dependent on its N-terminal amino acid, and therefore relate its half-life to the N-end rule pathway of targeted protein degradation (NERD), non-Methionine (Met) amino acids need to be exposed at their amino terminal in most cases. Per definition, at this position, destabilizing residues are generally unlikely to occur without further posttranslational modification of immature (pre-)proproteins. Moreover, almost exclusively, stabilizing, or not per se destabilizing residues are N-terminally exposed upon Met excision by Met aminopeptidases. To date, there exist two prominent protocols to study the impact of destabilizing residues at the N-terminal of a given protein by selectively exposing the amino acid residue to be tested. Such proteins can be used to study NERD substrate candidates and analyze NERD enzymatic components. Namely, the well-established ubiquitin fusion technique (UFT) is used in vivo or in cell-free transcription/translation systems in vitro to produce a desired N-terminal residue in a protein of interest, whereas the proteolytic cleavage of recombinant fusion proteins by tobacco etch virus (TEV) protease is used in vitro to purify proteins with distinct N-termini. Here, we discuss how to accomplish in vivo and in vitro expression and modification of NERD substrate proteins that may be used as stability tester or activity reporter proteins and to characterize potential NERD substrates.

The methods to generate artificial substrates via UFT or TEV cleavage are described here and can be used either in vivo in the context of stably transformed plants and cell culture expressing chimeric constructs or in vitro in cell-free systems such as rabbit reticulocyte lysate as well as after expression and purification of recombinant proteins from various hosts.

Key words N-end rule pathway, Ubiquitin fusion technique, TEV protease, N-terminomics, Protease, Degradomics

1 Introduction

The abundance and activity of all cellular proteins, the proteome, have to be strictly regulated to ensure their proper function. Proteostasis control is accomplished on transcriptional, translational, and posttranslational levels. One of these protein quality control checkpoints is the ubiquitin-proteasome system (UPS), a part of the cellular protein modification machinery utilizing the small protein modifier ubiquitin (Ub), which can lead to the

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degradation of, e.g., misfolded proteins or of those which function is either not any longer needed for cell viability or even may cause cytotoxic effects.

The N-end rule degradation pathway (NERD) of targeted proteolysis is a specialized part of the UPS, *see* Fig. 1a. It links the half-life of a protein to its N-terminal amino acid and is built up in a hierarchical way comprising—for some substrates—a multi-step biochemical reaction cascade involving several highly specific

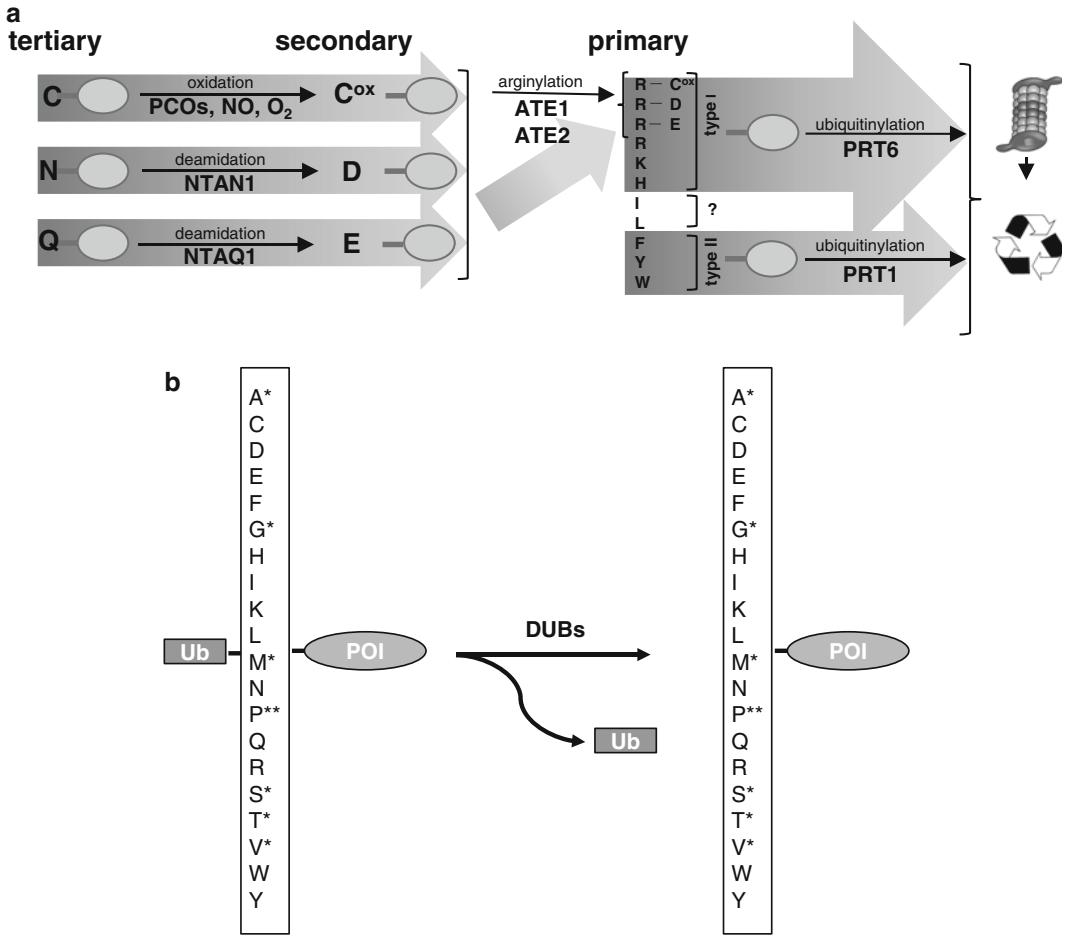

Fig. 1 N-end rule degradation pathway (NERD) of targeted proteolysis and Ubiquitin fusion technique (UFT). (**a**) NERD in plants. Substrates bearing an N-terminal primary, secondary, or tertiary destabilizing residue can be recognized and enzymatically modified by amidases (NTAs), arginyl transferases (ATEs), and NERD E3 Ub ligases (PRTs). Cys can be nonenzymatically oxidized by reactive oxygen species (1–4); or enzymatically by plant cysteine oxidases (PCOs; [15]), (**b**) UFT and amino acids possible to engineer at the N-terminus. UFT allows in vivo generation of needed N-termini. Ub-POI fusion proteins expressed are deubiquitinated via DUBs. After Ub removal amino acid in position 1 is exposed. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. *: amino acids not per se destabilizing, **: Ub-Pro is slowly processed by DUBs [25]

protein modifying enzymes. NERD function is conserved among all kingdoms albeit involving various ways to accomplish proteolysis, i.e., either enzymes of the eukaryotic UPS or bacterial or organellar proteases [1–5]. A protein bearing a destabilizing N-terminal such as basic, bulky or hydrophobic side chains, in this context known as N-degrons, which may occur as possible protein cleavage products can be recognized via NERD E3 Ub protein ligases—the so called N-recognins—followed by polyubiquitination and degradation by the 26S proteasome. Both steps are regulated in yeast and animals by functional homologs of the Ubr1 E3 Ub protein ligase [2–4]. Proteins bearing a tertiary destabilizing residue (Cys, Asn, Gln) can be modified in an enzymatic (deamidation of Asn and Gln by N-terminal amidases (NTAs) or oxidation of Cys) or nonenzymatic (oxidation of Cys by reactive oxygen species (ROS) such as nitric oxide (NO)) reaction to become secondary destabilizing residues (oxidized Cys [Cys^{ox}], Asp, Glu). These amino acids can be recognized via tRNA-arginyltransferases (ATEs) that attach an Arg residue as a primary destabilizing side chain to their substrates (Fig. 1a).

Known NERD substrates in yeast and animals are proteins and peptide fragments with mainly regulatory functions but also related to human diseases such as Alzheimer's and Parkinson's [6]. In plants, only five proteins, i.e., members of the group 7 ethylene response factors (ERFs), are associated with NERD-mediated degradation [7–9]. These targets are transcription factors involved in water–stress response. In their specific case, the proteins start with Met followed by Cys (MC-starting proteins). Met is rapidly cleaved off by MAPs if the second amino acid is stabilizing, e.g., Gly or not per se destabilizing, Cys. Under normoxic conditions, the Cys is oxidized and the proteins are degraded via NERD. Under hypoxic conditions, the absence of oxygen leads to their stabilization. Other proteins are discussed as NERD substrates but not verified until now.

In *Arabidopsis*, the two Ubr1 functional homologs PROTEOLYSIS (PRT) 1 [10, 11] and 6 [12], the two arginyltransferases ATE1 and 2 [13, 14], as well as plant cysteine oxidases (PCOs) [15] are known NERD components but their physiological role and molecular mechanisms are largely unknown. Additionally, PRT1 is—as a plant pioneer protein—completely unrelated to known Ubr enzymes [6]. Plant NERD seems to be mechanistically diverse due to nonhomologous ATEs and the mild phenotype of plant *prt* mutants hints toward the existence of further important NERD enzymes showing lower sequence similarities with known homologs.

To further relate plant NERD to biological functions, it is pivotal to identify substrates of the different enzymatic components and the entire degradation pathway.

1.1 Importance of Creating Proteins Comprising Defined N-Termini

To analyze the stability of NERD substrate candidate proteins, it is inevitable to use methods that allow creation of distinct non-Met N-termini of choice for studies both in vivo and in vitro. The need of specific and exposed N-termini as a starting point for protein stability assays and testing of hypotheses becomes clear from Fig. 1a, where the questionable residues are ordered according to their possible influence on protein stability. Generation of the mentioned non-Met N-termini is not possible applying regular translation as all open reading frames, regardless of transcribed and translated in vivo or in vitro, need to commence with a start codon. Therefore, freshly synthesized proteins will necessarily contain the initiator Met residue. In vivo, in most cases, cotranslational N-terminal Met excision (NME) by MAPs leads to the cleavage of the initiator Met residue if the second amino acid is not a primary, secondary or tertiary destabilizing one according to NERD or another Met. One exception is Cys which may be presented at the N-terminal after NME [16, 17]. However, Met-Cys-starting proteins are highly underrepresented, e.g., in the *Arabidopsis* proteome. Particularly, in vivo and in vitro studies demonstrated that MAPs remove the initiator Met only if the second residue has a small radius of gyration of the side chain. Thus, bulky amino acids do not allow Met removal [18].

NME occurs at the Met adjacent to residues at position two of the nascent protein which are classified as stabilizing residues—or as not per se destabilizing residues—such as Ala, Gly, Val, Ser, and Thr but also Pro and, as mentioned above, Cys. Met is retained at the N-terminal if the second residue is another Met [19–22]. We recently confirmed this Met excision "dogma" and the underrepresentation of charged and hydrophobic amino acids at the N-terminal also for *Arabidopsis* [23].

If proteins comprise N-terminal signal or transit peptides, they can be cleaved off after transport into the desired compartment, e.g. ER, nucleus, mitochondria, or chloroplasts. Many proteins, especially zymogenes, i.e., precursors of enzymes, are also translated as (pre-)proproteins or (pre-)propeptides bearing an N-terminal sequence which is cleaved off autocatalytically or by a protease. One example is the formation of active trypsin [24].

To characterize the stability of a protein of interest (POI) dependent on the N-terminal, it has to be made sure that the protein expressed in vivo or in vitro is bearing the N-terminal amino acid in question to which the half-life is to be correlated to. There exist two ways to artificially circumvent this problem for experimental purposes and produce test proteins in a way that they can be metabolized by NERD, i.e., expression of chimeric proteins via the so-called ubiquitin fusion technique (UFT) and, cleavage after recombinant production by the catalytic domain of the Nuclear Inclusion a (NIa) protein encoded by the tobacco etch virus (TEV) to generate distinct N-termini of proteins for further

characterization. In the following, we explain three experimental strategies how to use UFT in vivo, UFT in vitro, and TEV cleavage to create test proteins in this context.

1.2 Ubiquitin Fusion Technique

UFT was originally established in *S. cerevisiae* to study protein stability according to a distinct N-terminal [25]. It was extensively used to characterize NERD substrates and enzymatic components [26, 27]. UFT is based on the natural occurrence and processing of the small protein Ub which is synthesized as a polymer [28] followed by cleavage into single Ub moieties after its last residue Gly76 by deubiquitinating enzymes (DUBs) [29, 30]. UFT was initially used to express Ub-X-β-galactosidase constructs in *S. cerevisiae* and *E. coli*. Only in the yeast, the fusion was cleaved by DUBs as the bacteria are lacking this machinery and the UPS [25]. UFT allows "automatic" cleavage if DUBs are present and it works for all amino acids in position X albeit the cleavage of Ub-Pro is not very efficient [25, 27, 30]. It is also possible to use UFT in Ub-lacking prokaryotes but it is necessary to cotransform, e.g., the yeast DUB Ubp1 which is then able to remove Ub from fusion proteins in the bacterial host [31].

We use this cotranslational cleavage mechanism to separate a Ub-X-POI fusion protein, with X being the wanted N-terminal amino acid. Also in this artificial context, the fusion is cotranslationally cleaved after Gly76 of Ub and the resulting C-terminal fragment of the fusion protein showing the engineered N-terminal (Fig. 1b).

1.3 UFT In Vivo

UFT allowed screening for mutants with impaired proteasomal degradation of proteins bearing a distinct N-terminus. In *Arabidopsis*, the E3 Ub ligase PRT1 was identified by a forward genetics screen based on Ub-F-DHFR reporter constructs. The F-construct was found to be stabilized in *prt1* mutant plants [10, 32]. Also the second identified plant NERD E3 ligase was validated using UFT [12]. Here, the Ub reference technique (URT) was used where the Ub is N-terminally tagged with a second reporter protein to follow fusion protein cleavage with an internal reference DHFR-Ub^{K48R} [27]. DHFR-Ub^{K48R} contains a mutated Ub to prevent its recognition as degradation signal of the Ub fusion degradation pathway (UFD). In our experiments, we can use the wild type version of Ub as we use it without a reference. In *prt6* mutant plants harboring DHFR-Ub^{K48R}-X-β-galactosidase (X = M, L, F, R), the R-β-gal was stabilized whereas in the wild type this construct is highly instable [12].

We usually express Ub-X-POI-YFP (X = D, R, G) to study stabilization of the POI according to it's N-terminus in the in vivo system of plant protoplasts. This system allows to transiently transform plant material including cell types derived from mutant lines and study, e.g., protein localization, movement or interaction [33, 34].

mCherry is cotransformed as a transformation control and the ratio of protoplasts showing a YFP and an mCherry signal in comparison to protoplasts transformed only with mCherry is calculated. In parallel we analyze the protein abundance via western blotting.

1.4 UFT In Vitro

In order to study protein half-life in vitro, UFT can be used in mammalian cell culture [35], *Xenopus* oocyte extracts [36], and commercially available reticulocyte lysate [37]. The latter allows to easily study stability effects in vitro in an eukaryotic background comprising a functional proteasome. In the plant field, reticulocyte lysate independent of UFT was applied to study protein degradation of MC-starting proteins of the group 7 ethylene response factors (ERFs; [7]). Here, UFT is not required as MAPs cleave off the first Met and the following Cys is enzymatically oxidized by PCOs [15] or nonenzymatically by ROS [4]. The proteins can then be degraded via NERD. These ERFs are the only described NERD substrates in plants so far. The same reticulocyte lysate system was used to follow the degradation of RGS4 and 5 as substrates of mammalian NERD [37]. Here, the process was monitored with biotin-labeled proteins.

In the protocol outlined below, UFT fusion proteins are expressed under control of the T7 promoter and their stability was monitored via SDS-PAGE followed by western blot and immunostaining after cycloheximide treatment.

1.5 TEV Cleavage

Recombinant protein production is often accomplished by expressing tagged proteins or entire fusion proteins comprising larger accompanying non-POI protein moieties which serve to enhance expression and solubility or facilitate further downstreaming, e.g., enrichment, cleavage, and purification, of the fusion itself or its separated parts. Highly sequence-specific proteases enable the cleavage of such fusion protein tags or fusion protein partners from the actual target POI. To precisely remove an N-terminal tag from a fusion protein is the key for controlled exposure of a desired N-terminal of a given POI, e.g., in the context of studying the impact of recognition by N-recognins and other enzymatic NERD components. Among such highly specific and versatile proteases, TEV protease, i.e., the catalytic domain of the Nuclear Inclusion a (NIa) protein encoded by the TEV, gained popularity both in in vitro and in vivo applications due to its high sequence specificity, low enzymatic promiscuity, non-toxicity, relatively high catalytic turnover, insensitivity to many proteinase inhibitors and ease of removal after its action [38, 39] (*see* **Note 1**). In our studies, we use the recognition sequence of TEV as a linker between an N-terminal octahistidine-MBP (maltose binding protein, $(His)_8$-MBP) which used both as an affinity and purification tag and the X-POI, where X stands again for the N-terminal amino acid to be engineered. TEV is highly sequence-specific to its canonical

recognition site ENLYFQ|X, with X being the relevant P1′ amino acid and therefore the new N-terminally exposed residue of the POI [40]. TEV displays optimal performance on the peptide sequence ENLYFQ|S/G [41], where Q and S or G corresponds to the P1 and P1′ residues. P1′ is typically known to be G or S but can be successfully replaced by all known amino acids with no or little activity loss except for proline which drastically hindered the TEV recognition ability [40, 42, 43]. Recently, new proteases suitable as candidates for tag removal were described having much higher activity even at 0 °C with a broader optimal range for buffer and/or salt conditions [44].

In our case, we use a construct containing an N-terminal $(His)_8$-MBP fused to a Phe- or Gly-starting and C-terminally His-tagged substrate sequence as POI. This sequence is based on *E. coli* lacZ, known as eK (extension containing lysines/Ks) and has extensively been used to generate artificial NERD substrates to characterize this proteolytic pathway [25, 45]. $(His)_8$-MBP and eK are separated by a linker which contains a TEV cleavage site, as illustrated in Fig. 3a, b. The $(His)_8$-MBP tag facilitates purification and is easily removed by a TEV digest, allowing eK substrate generation and purification for X-eK-His (Fig. 4a, b). The F-eK-His is an excellently working artificial NERD substrate and was successfully tested in in vitro ubiquitination with G-eK-His as a negative control (Fig. 4c).

Examples for engineering various P1′ sites and substituting amino acids by replacing the G/S within the canonical TEV recognition site are Asp or Phe [42, 43, 46, 47], and Cys [48]. A new version of TEV protease almost completely lacks specificity for the amino acid at position P1′ and allows an even broader variety of N-terminals to be exposed via cleavage [49].

2 Materials

2.1 General Equipment

1. Climatized greenhouse or growth chambers.
2. Fridge or cold room for TEV preparation and cleavage of fusion proteins.
3. Cooling microcentrifuge or regular microcentrifuge in cold room.
4. 1.5-mL microcentrifuge tubes.
5. Flat-tip forceps and razor blade.
6. Dewar container with swimmer, liquid nitrogen.
7. Ice bucket, wet ice.
8. Vortex mixer.
9. Graduated cylinders and containers for reagent preparation and storage.
10. Pipets accurately delivering 2.5, 20, 200, and 1000 µL.

11. Soil mixture for *Arabidopsis* cultivation, selection and propagation: steamed (pasteurized for min. 3 h at 90 °C) soil mixture of Einheitserde Classic Kokos (45% (w/w) white peat, 20% (w/w) clay, 15% (w/w) block peat, 20% (w/w) coco fibers; cat. no. 10-00800-40, Einheitserdewerke Patzer, Gebr. Patzer); 25% (w/w) Vermiculite (grain size 2–3 mm; cat. no. 29.060220, Gärtnereibedarf Kamlott), 300–400 g/m^3 soil substrate of Exemptor (100 g/kg thiacloprid, cat. no. 802288, Hermann Meyer).
12. Confocal laser scanning microscope with 514 and 587 nm excitation wavelength, emission filters for 509 and 610 nm.

2.2 Cloning

1. cDNA from *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia-0 (Col-0).
2. Standard cloning equipment.
3. Site-specific oligonucleotides (*see* Table 1).
4. Proofreading polymerase such as Pfu.
5. Gateway BP and LR kits (Invitrogen).
6. Gateway-compatible Entry and Destination vectors (*see* Table 2).
7. DH5α or equivalent cloning hosts (Invitrogen).
8. *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene/Agilent) or equivalent bacterial expression hosts.
9. Gel extraction Kit (Thermo Scientific, K0513).
10. DNA Maxi-Prep Kit (Macherey & Nagel NucleoBond, PC 500).

Table 1
Oligonucleotide primers used for PCR

Primer name	Sequence (5′–3′)	Annealing temperature (°C)
Sequences for UFT constructs		
ss_attB1_Ub	GGGGACAAGTTTGTACAAAAAAGCAG GCTTAGCCGCCACCATGCAGATCTTCGTCAAG	51
ss_bridge_Ub_attB1	ACCATGCAGATCTTCGTCAAGACGTTAAC	56
as_Ub_POI	[NNN]*n*CCCACCTCTAAGTCTTAAGACAAGATG	Depending on GOI
ss_Ub_POI	GTGGG[NNN]*n*	Depending on GOI
as_POI	[NNN]*n*	Depending on GOI
Sequences for TEV constructs		
ss_adapter_tev	GGGGACAAGTTTGTACAAAAAAGCA GGCTTAGAAAACCTGTATTTTCAG	48
ss_tev_X_POI	GCTTAGAAAACCTGTATTTTCAG**XXX**[NNN]*n*	Depending on GOI
as_attB2_POI	GGGGACCACTTTGTACAAGAAAGCTGGGTA[NNN]*n*	Depending on GOI

NNN: bases specific for annealing on DNA sequence encoding POI, XXX: codon for N-terminal amino acid residue of interest, GOI: gene of interest encoding POI

Table 2
Vectors used

Vector	Description	Source or reference
pDONR201	Gateway donor vector for single Gateway recombination (attP1/P2)	Invitrogen
pVP16	Gateway destination vector (attR1/R2) containing a 8×His:MBP coding sequence 5′ of the Gateway cassette leading to an N-terminal 8×His:MBP double affinity tag under control of Pro_{T5} and a Lac-Operon for protein induction (*see* **Note 11**). bla resistance in bacteria; Gateway recombinational cloning into this vector removes ccdB and cat	Kind gift of Russell L. Wrobel, Protein Structure Initiative (PSI) at the Center for Eukaryotic Structural Genomics (CESG), University of Wisconsin-Madison [54]
pOLENTE	Gateway.compatible destination vector (attR1/R2) for coupled transcription/translation, based on pTNT (Promega). bla resistance in bacteria; Gateway recombinational cloning into this vector removes ccdB and cat	Details on the cloning will be published elsewhere
pUBC-YFP	Gateway destination vector (attR1/R2) containing a YFP coding sequence 3′ of the Gateway cassette leading to a C-terminal YFP. Fusion protein is under control of the *Arabidopsis* Ubiquitin-10 (At4g05320) promotor. bla resistance in bacteria; Gateway recombinational cloning into this vector removes ccdB and cat	[55]
pRK793	pRK793 overproduces the TEV catalytic domain as an MBP fusion protein that cleaves itself in vivo to yield a TEV protease catalytic domain with an N-terminal His-tag and a C-terminal polyarginine tag, plasmid based on pMal-C2 (New England Biolabs).	pRK793 was a gift from David Waugh (Addgene plasmid # 8827; [53])
pVP16-tev-POI	Gateway expression vector (attB1/B2) based on pVP16 comprising a primer-born TEV recognition site (ENLYFQ-X) at the junction to the POI	This work

bla: β-lactamase, ccdB: cell death cassette, cat: chloramphenicol acetyltransferase

2.3 Protoplast Isolation (See Table 3)

1. 5 M sodium chloride (NaCl).
2. 1 M potassium chloride (KCl).
3. 0.1 M calcium chloride ($CaCl_2$).
4. 0.8 M mannitol (sterile-filtered).
5. 0.2 M MES (4-morpholineethanesulfonic acid; pH 5.7).
6. Haemocytometer.
7. Pre-cut 1000 µL pipet tips.
8. Macerozyme R10 (SERVA).
9. Cellulase R10 (SERVA).

Table 3
Buffers and solutions used for protoplast isolation and transformation

All buffers except W5 (storable for 1 week at 4 °C) have to be prepared freshly.									
Buffer W5									
Component	Stock conc.	Final conc.	50 mL	100 mL	200 mL	400 mL	500 mL		
NaCl	5 M	154 mM	1.54	3.08	6.16	12.32	15.4	mL	
$CaCl_2$	1 M	125 mM	6.25	12.5	25	50	62.5	mL	
KCl	0.1 M	5 mM	2.5	5	10	20	25	mL	
MES pH 5.7	0.2 M	2 mM	0.5	1	2	4	5	mL	
H_2O			39.21	78.42	156.84	313.68	392.1	mL	
Buffer WI									
Component	Stock conc.	Final conc.	5 mL	10 mL	15 mL	20 mL	30 mL	40 mL	
Mannitol	0.8 M	0.5 mM	3.15	6.3	9.45	12.6	18.9	25.2	mL
KCl	0.1 M	20 mM	1	2	3	4	6	8	mL
MES pH 5.7	0.2 M	4 mM	0.1	0.2	0.3	0.4	0.6	0.8	mL
H_2O			0.75	1.5	2.25	3	4.5	6	mL
Buffer MMG									
Component	Stock conc.	Final conc.	5 mL	10 mL	15 mL	20 mL	30 mL	40 mL	
Mannitol	0.8 M	0.4 M	2.5	5	7.5	10	15	20	mL
$MgCl_2$	0.15 M	15 mM	0.5	1	1.5	2	3	4	mL
MES pH 5.7	0.2 M	4 mM	0.1	0.2	0.3	0.4	0.6	0.8	mL
H_2O			1.9	3.8	5.7	7.6	11.4	15.2	mL
PEG solution									
Component	Stock conc.	Final conc.	5 mL	10 mL	15 mL	20 mL	30 mL	40 mL	
Mannitol	0.8 M	0.2 M	1.25	2.5	3.75	5	7.5	10	mL
$CaCl_2$	1 M	0.1 M	0.5	1	1.5	2	3	4	mL
PEG	Solid	40%	2	4	6	8	12	16	g
H_2O			1.5	3	4.5	6	9	12	mL
Enzyme solution									
Component	Stock conc.	Final conc.	5 mL	10 mL	15 mL	20 mL	30 mL	40 mL	
Mannitol	0.8 M	0.4 M	2.5	5	7.5	10	15	20	mL
KCl	0.1 M	20 mM	1	2	3	4	6	8	mL
MES pH 5.7	0.2 M	20 mM	0.5	1	1.5	2	3	4	mL
H_2O			0.95	1.9	2.85	3.8	5.7	7.6	mL
Cellulose R10		1.50%	75	150	225	300	450	600	mg
Macerozyme R10		0.40%	20	40	60	80	120	160	mg
$CaCl_2$	1 M	10 mM	50	100	150	200	300	400	µL
BSA	0.1 g/mL	1 mg/mL	50	100	150	200	300	400	µL

10. BSA (Roth).
11. Sheet of regular printer paper.
12. Desiccator.
13. Black cloth (for desiccator).
14. Nylon mesh (100 µm mesh size).
15. Cell culture tubes (polystyrene, sterile, with screw cap; Greiner).

2.4 PEG-Mediated Transformation of DNA into Protoplasts (See Table 3)

1. 150 mM magnesium chloride ($MgCl_2$).
2. Polyethylene glycol (PEG) 4000.
3. 1 M potassium chloride (KCl).
4. 0.1 M calcium chloride ($CaCl_2$).
5. 0.8 M mannitol (sterile-filtered).
6. 0.2 M MES (4-morpholineethanesulfonic acid; pH 5.7).

2.5 Analysis of Protein Expression in Protoplasts

1. 1× SDS-loading buffer: 50 mM Tris–Cl (pH 6.8), 50 mM DTT, 1 % (v/v) SDS, 10 % (v/v) glycerol, 0.01 % (v/v) bromophenol blue.
2. Pre-cut 1000 µL pipet tips.

2.6 Protein Expression in a Cell-Free System

1. TNT T7 Coupled Reticulocyte Lysate System (Promega, L4610).
2. 0.5 M cycloheximide (Sigma).
3. 1× SDS loading buffer as in Subheading 2.5, **item 1**.

2.7 Protein Expression in Bacteria

1. LB medium (Roth).
2. 500 mL Erlenmeyer flasks.
3. 1 M IPTG (isopropyl β-D-1-thiogalactopyranoside).
4. 100 mM PMSF (phenylmethanesulfonylfluoride).
5. Ni-buffer: 100 mM Tris–Cl pH 8, 300 mM NaCl, 0.25 % (v/v) Tween, 10 % (v/v) glycerol.
6. Ni-elution buffer: 100 mM Tris–Cl pH 8, 300 mM NaCl, 0.25 % (v/v) Tween, 10 % (v/v) glycerol, 200 mM imidazole.
7. Amylose buffer: 20 mM Tris–Cl pH 7.4, 200 mM NaCl, 1 mM EDTA.
8. Amylose elution buffer: 20 mM Tris–Cl pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM maltose.
9. Ni-NTA agarose (nickel-charged resin; Qiagen).
10. Amylose agarose (amylose resin; NEB).
11. Polypropylene columns (5 mL; Qiagen).
12. 40 g/mL lysozyme (Sigma) in Ni-buffer.
13. Imidazole (Merck).
14. Maltose (Roth).
15. Appropriate antibiotics (in our case, a stock solution of 50 mg/mL carbenicillin).

2.8 TEV Cleavage

1. Home-made TEV protease (*see* **Note 2**).
2. TEV reaction buffer (50 mM Tris–HCl pH 8, 0.5 mM EDTA, 1 mM DTT).

3. Ni-buffer: 100 mM Tris–HCl pH 8, 300 mM NaCl, 0.25% (v/v) Tween, 10% (v/v) glycerol.
4. Ni-elution buffer: 100 mM Tris–HCl pH 8, 300 mM NaCl, 0.25% (v/v) Tween, 10% (v/v) glycerol, 200 mM imidazole.
5. Amylose buffer: 20 mM Tris–HCl pH 7.4, 200 mM NaCl, 1 mM EDTA.
6. Amylose-elution buffer: 20 mM Tris–Cl pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM maltose.
7. Ni-NTA agarose (nickel-charged resin; Qiagen).
8. Amylose resin (NEB).
9. Polypropylene columns (5 mL Qiagen).
10. Imidazole (Merck).
11. Maltose (Roth).
12. Amicon Ultra-15 (Merck Millipore; 30 and 10 kDa cut-off).

2.9 Protein Concentration Determination

1. Protein Assay Dye Reagent Concentrate (Bio-Rad).
2. 1 mg/mL bovine serum albumine (BSA).
3. Microplate reader.
4. Spectrophotometer set to 595 nm.

2.10 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. SDS-PAGE equipment and power supply, we preferentially use small gel systems such as a BioRAD.
2. 30% acrylamide mix (30% Acrylamide/*N*,*N*′-Methylenebisacrylamide solution, ratio 37.5:1 in water, *see* **Note 3**).
3. 1.5 M Tris–HCl pH 8.8 (for separating gel).
4. 1 M Tris–HCl pH 6.8 (for stacking gel).
5. 10% (w/v) Ammonium persulfate (APS, immediately freeze upon preparation in single use aliquots, store at −20 °C).
6. 10% (w/v) sodium dodecyl sulfate (sodium lauryl sulfate, SDS).
7. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED, *see* **Note 4**).
8. Isopropanol (*see* **Note 5**).
9. Gel loading tips, extended length.
10. Protein gel running buffer 10×; for 1 L: 30.2 g Tris base, 144.2 g glycine, and 10 g SDS in water.
11. Prestained molecular weight markers such as Precision Plus Protein Standard All Blue (Bio-Rad).
12. 5× SDS sample buffer: 0.25 M Tris-HCl pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 0.25 M DTT [50]. For 1× SDS Sample buffer add 10 µL of 5× SDS sample buffer to 40 µL of water.

13. Prepare gel volumes according to the sizes of your gel system, all percentages in (v/v), modified from [51]:
    (a) For a 12% separating gel add 33% water, 40% acrylamide mix (30%), 25% 1.5 M Tris–Cl pH 8.8, 1% SDS (10% (w/v)) and APS (10% (w/v)), and 0.04% (v/v) TEMED.
    (b) For a 5% stacking gel add 68% water, 17% acrylamide mix (30%), 12.5% 1.5 M Tris–Cl pH 8.8, 1% SDS (10% (w/v)) and APS (10% (w/v)), and 0.1% (v/v) TEMED.

2.11 Western Transfer of Proteins and Detection

1. Semi-dry blot apparatus (such as BioRAD Trans-Blot SD Semi-Dry Transfer Cell).
2. Power supply.
3. Filter paper (Whatman).
4. PVDF membrane (GE Healthcare).
5. methanol for activation of PVDF membrane.
6. 3% (w/v) dry milk in TBST.
7. 5% (w/v) dry milk in TBST.
8. Tris-buffered saline (TBS) 10× for 1 L: solve 87.66 g of NaCl and 12.11 g of Tris-base in water; adjust to pH 7.5.
9. TBST for 1 L: add 100 mL of 10× TBS (for 150 mM NaCl and 10 mM Tris–Cl pH 8.0), 10 mL of 10% (v/v) Tween 20 (for 0.1% (v/v)) to water.
10. 10× semi-dry transfer buffer: for 1 L, add 58 g of Tris base (for 47 mM) and 29 g of glycin (for 50 mM) in water. 1× semi-dry transfer buffer: for 1 L, add 100 mL of 10× buffer and 200 mL of methanol to 1 L with water.
11. Saran wrap or plastic disposal bags to wrap membranes during ECL detection.
12. Enhanced chemiluminescent reagents for chemiluminescent imaging (ECL, SuperSignal West Femto, Pierce cat. no. 1858415).
13. BioMax Light Film for chemiluminescent imaging (Hartenstein).
14. Autoradiography cassette, film, and film developing unit.
15. Antibodies used for protoplast, reticulocyte lysate and recombinant protein work are listed in Table 4.

3 Methods

3.1 General Considerations

Here we outline how we use UFT and TEV fusions to study protein stability and degradation. Time considerations for UFT in protoplasts are 2–3 days and for UFT in reticulocyte lysate

Table 4
Antibodies used for protoplast, reticulocyte lysate and recombinant protein work

Antigen	Species, type	Name	Supplier	Cat. No.	Conditions	Range of use		
						Protoplast lysate	Reticulocyte lysate	Recombinant protein
1° antibodies								
HA tag	Mouse, monoclonal	HA.11	Covance or HISS	MMS-101	1:1000 dilution in TBST 4% milk	Yes	Yes	Yes
HA tag	Rabbit, polyclonal	HA-probe (Y-11)	Santa Cruz Biotechnology	sc-805	Western blot 1:200 dilution in TBST 3% milk	No	Not tested	Yes
His tag	Rabbit, polyclonal	His-probe (H-15)	Santa Cruz Biotechnology	sc-803	Western blot 1:200 dilution in TBST 3% milk	No	Not tested	Yes
His tag	Mouse, monoclonal	Anti-His antibody	GE Healthcare	27-4710-01	Western blot 1:1000 dilution in TBST 3% milk	Yes	Yes	Yes
Green fluorescent protein (GFP)	Rabbit, polyclonal	GFP (FL)	Santa Cruz Biotechnology	sc-8334	1:1000 dilution in TBST 4% milk	Yes	Not tested	Yes
Green fluorescent protein (GFP)	Mouse, monoclonal	GFP (B-2)	Santa Cruz Biotechnology	sc-9996	Western blot 1:1000 dilution in TBST 3% milk	Not tested	Not tested	Yes
Ubiquitin	Mouse, monoclonal	Ub (P4D1)	Santa Cruz Biotechnology	sc-8017	Western blot 1:1000 dilution in TBST 3% milk	No	Not tested	Yes
2° antibodies								
Mouse	Goat, IgG	HRP-conjugated antibody Immuno Pure Peroxidase	Pierce	31430	Western blot 1:5000 dilution in TBST 3% milk	Yes	Yes	Yes
Mouse	Goat, IgG	Anti-mouse IgG-HRP	Santa Cruz Biotechnology	sc-2005	Western blot 1:5000 dilution in TBST 3% milk	Not tested	Not tested	Yes
Rabbit	Goat, IgG	HRP-conjugated antibody Immuno Pure Peroxidase	Pierce	1858415 NCI8415	Western blot 1:5000 dilution in TBST 3% milk	Yes	Yes	Yes
Rabbit	Goat, IgG	Anti-rabbit IgG-HRP	Santa Cruz Biotechnology	sc-2004	Western blot 1:5000 dilution in TBST 3% milk	Not tested	Not tested	Yes

1–2 days, each starting from either transformation or transcription/translation. After stratification of 4–5 days at 4 °C in the dark, *Arabidopsis* seeds were germinated and plants grown under standard short day (8/16 h light/dark) greenhouse conditions.

3.2 Cloning

We applied classical PCR cloning strategies to obtain the chimeric DNA fusions containing *Arabidopsis* GOIs. In brief, primers containing the Gateway attB1 and attB2 sites (Table 1) were used for flanking both the 5′- and 3′-ends of each final fusion construct. This protocol was based on subsequent two-step fusion PCR and is explained in the following.

ORFs to express fusion proteins for UFT were cloned in a two step PCR. Ub was cloned using primer pair (ss_bridge_Ub_attB1/as_Ub_POI) from vector template DNA originally based on a synthetic human Ub gene (pRTUB8; [32, 52]).

In parallel, the GOI was cloned with an overhang to Ub with primers (ss_Ub_POI/as_attB2_POI) from cDNA (Fig. 2).

The fragments were purified via gel extraction and afterwards fused using primers (ss_attB1-Ub/as_attB2_POI) in a second PCR reaction (56 °C annealing temperature, 1 min extension time).

For attB1-tev-POI-attB2 fragments, the GOI was cloned from cDNA using primer ss_tev_X_POI as sense primer (X = N-terminal amino acid after TEV-cleavage of fusion protein). To complete the attB1 site, a second PCR with ss_adapter_TEV as sense primer was done (Fig. 3). The obtained fragments were recombined into pDONR201 vector using Gateway BP Clonase enzyme mix (Invitrogen) and analyzed via restriction digest and sequencing, primers can be found in Table 1. The insert of the resulting Entry vector was recombined into the respective destination vector (pVP16 for tev-constructs, pUBC-YFP for protoplasts, pOLENTE for the cell-free system) (Figs. 2 and 3). The isolated Expression vector was used to the respective experiment.

3.3 Protoplast Isolation

Preparation of protoplasts was done as described previously [33] and we only highlight some differences in our protocol.

All solutions except W5 need to be prepared freshly according to the needed volumes. W5 can be stored in the fridge. Solubilisation of PEG requires often more than 2 h, therefore, it is necessary to start with it in the beginning of the preparations.

1. Enzyme solution should be prepared as follows: Add MES, mannitol and KCl and preheat the solution to 55 °C. Now add the enzymes and keep the temperature for 10 min at 55 °C. Then, cool on ice and add $CaCl_2$ and BSA. Filter the solution through a 45 μm acetate filter into a Petri dish or similar.
2. Choose well-expanded leaves from 5- to 8-week-old plants grown in a stress-free environment under standardized conditions.

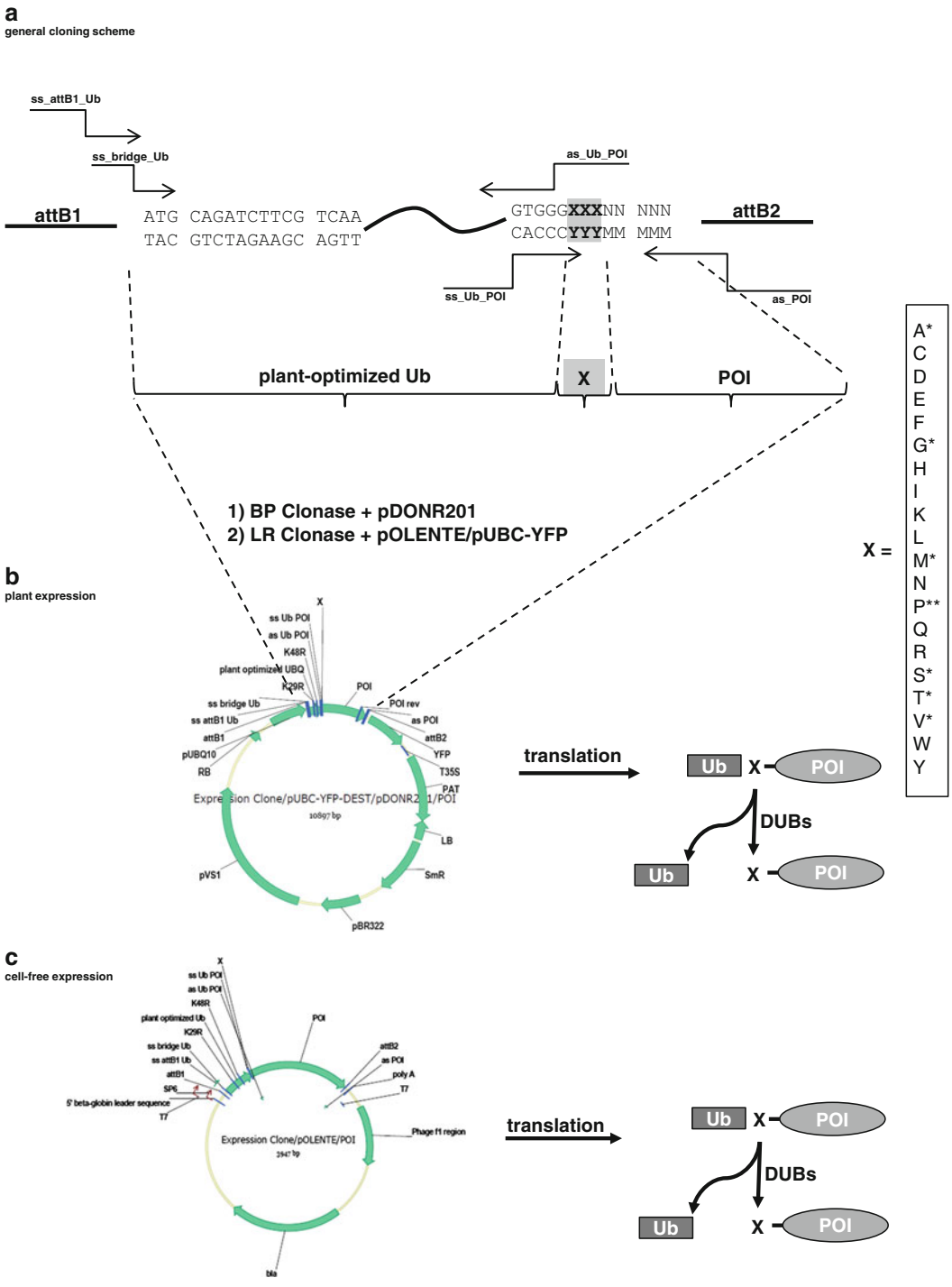

Fig. 2 Preparation of UFT constructs. (**a**) General cloning strategy. POI and Ub are subcloned from template DNA, PCR reactions are done as described in Subheading 3. The PCR fusion attB1-Ub-X-POI-attB2 is recombined into pDONR201 using Gateway BP clonase, followed by LR reaction into the designated destination vector. (**b**, **c**) Expression vectors and formation of X-POIs. (**b**) Cloning strategy for in vivo UFT. pUBC-YFP-Ub-X-POI for plant expression contains a Ub-POI-YFP fusion under control of a Ub promotor. After expression in planta, Ub is removed by DUBs and X is exposed as N-terminus. (**c**) Cloning strategy for in vitro UFT. pOLENTE-Ub-X-POI for in vitro expression in reticulocytes contains Ub-X-POI under control of the T7 promotor. After translation, Ub is removed by DUBs and X is exposed as N-terminal. *: amino acids not per se destabilizing, **: Ub-Pro is slowly processed by DUBs

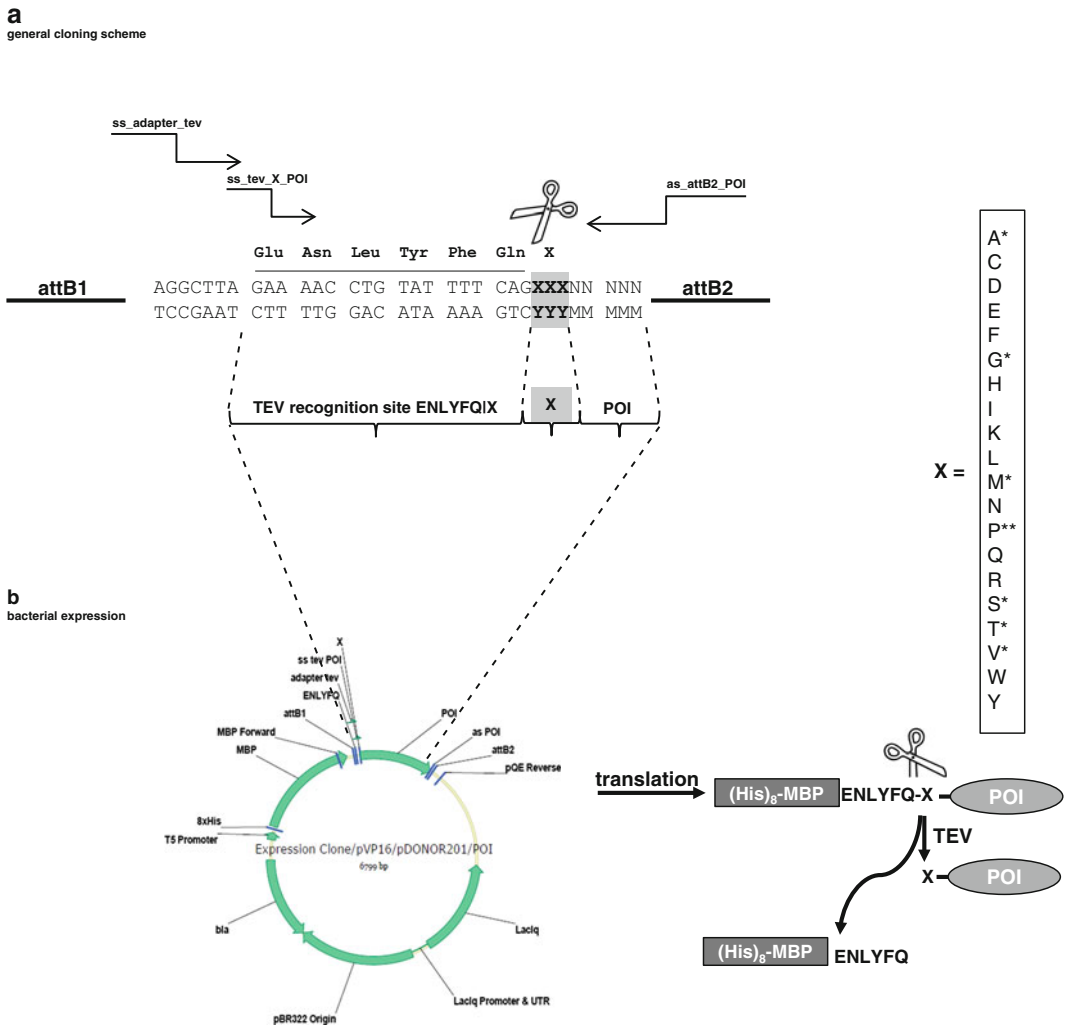

Fig. 3 Preparation of TEV cleavable fusion proteins. (**a**) General cloning strategy. GOI is cloned from cDNA with annotated primers containing the TEV recognition sequence ENLYFQ (tev) directly followed by X-POI. PCR reactions are done as described in Subheading 3. The PCR fusion attB1-tev-X-POI-attB2 is recombined into pDONR201 using Gateway BP clonase, followed by LR reaction into pVP16. (**b**) Expression vector and formation of X-POIs. pVP16-tev-POI is expressed in bacteria to obtain N-terminally tagged $(His)_8$-MBP-tev-POI. After purification, the $(His)_8$-MBP tag is removed by TEV cleavage as described in Subheading 3. *: amino acids not per se destabilizing, **: Ub-Pro is slowly processed by DUBs

3. For cell wall lysis, leaves can be cut individually or two to three leaves piled up. Prepare a clean printer paper sheet or similar to cut the leaves on.
4. Remove the top part and leaf stalk with a razor blade. Cut <0.5 mm leaf strips from the middle part of a leaf.
5. As soon as a leaf is cut into strips, transfer them into the prepared enzyme solution (around nine leaves in 3 mL of enzyme

solution in a Petri dish (3.5 cm)). Dip them completely into the solution by using a pair of flat-tip forceps.

6. Vacuum-infiltrate leaf strips for 30 min in the dark using a desiccator (covered with a black cloth).
7. Continue the digestion, in the dark for at least 3 h at RT. Best worked 20–22 °C, which are stable in an air-conditioned room.
8. Gently shake the enzyme solution to release the protoplasts. The solution should turn green and at least half of the leaf strips become transparent.
9. Filter the suspension through a nylon mesh (100 μm mesh size) into 12 mL pre-cooled cell culture tubes (polystyrene, sterile, with screw cap, Greiner). Keep tubes on ice.
10. Centrifuge the protoplast suspension for 1 min at 200 g at 4 °C and remove as much supernatant as possible.
11. Wash protoplasts with 2 mL of W5. Resuspend them by gently inverting the tubes.
12. Invert tube and take up 8 μL of the suspension to determine protoplast concentration using a haemocytometer (Always cut the tip ends when pipetting protoplasts to avoid damage).
13. Calculate the required volume of MMG solution to have a working concentration of 2×10^5 pp/mL (pp: protoplasts) for transformation (*see* **Note 6**).
14. Leave protoplasts on ice for 40 min. They will settle on the bottom of the tube by gravity.
15. Remove supernatant from protoplast pellet and do a second wash with 2 mL of W5. Let them rest for another 40 min.

3.4 PEG-Mediated Transformation of DNA into Protoplasts

1. During the sedimentation (**step 15** in Subheading 3.3), prepare either microcentrifuge tubes (for transformation of 100 or 200 μL of protoplast suspension, e.g., for fluorescence microscopy) or cell culture tubes (for transformation of 300–800 μL of protoplast suspension, e.g., for western blot analysis).
2. Add the required amount of plasmid DNA for transformation (10 μg of plasmid DNA/100 μL of protoplast suspension). Release the DNA at the bottom of the tubes to make sure successful transfer of the sample. For western blotting we normally use 300 μL of protoplast suspension.
3. Remove supernatant from the protoplast pellet and resuspend in the calculated volume of MMG solution at room temperature (20–22 °C) to get a concentration of 2×10^5 pp/mL. Mix gently by inverting.
4. Add protoplasts to plasmid DNA and mix gently by briefly inverting the tubes.

5. Add 1.1 protoplast suspension volumes of PEG solution to the tube and mix gently by inverting the tubes. Do one to two tubes at a time.
6. Incubate at room temperature (20–22 °C) for 5–10 min.
7. Add 4.4 protoplast suspension volumes of W5 to stop the transformation process. Mix by gently inverting the tubes.
8. Centrifuge for 1 min at 200×*g* (4 °C) and remove as much supernatant as possible.
9. Add 1 protoplast suspension volumes of W1, mix by gently inverting the tubes.
10. Place tubes horizontally and incubate in the dark at room temperature (20–22 °C) over night.

3.5 Analysis of Protein Expression in Protoplasts via Fluorescence Microscopy

1. Carefully transfer 300 µL of protoplast suspension to a fresh 1.5 mL microcentrifuge tube using a cut 1000 µL pipet tip.
2. After overnight expression, YFP abundance can be checked using a laser scanning microscope. YFP has an excitation wavelength of 514 nm and an emission wavelength of 532 nm. mCherry is excited by 587 nm and emits a signal at 610 nm.
3. To calculate the stability of the POI dependent on its N-terminus, in each experiment, the number of transformed protoplasts was counted with both fluorescence signals (YFP and transformation control mCherry) in relation to just mCherry expressing protoplasts.

3.6 Analysis of Protein Expression in Protoplasts by Western Blotting

1. Spin down shortly at maximum speed, discard supernatant and add 12 µL of 1× SDS loading buffer.
2. Heat for 2–10 min at 96 °C.
3. Load to a 12% SDS-PAGE gel. In order to load the entire sample, use combs for broad pockets, e.g., a ten-sample comb, for SDS-PAGE and western blotting, see below.

3.7 Protein Expression in a Cell-Free System

To express proteins in a cell-free system, we use the TNT T7 Coupled Reticulocyte Lysate system (Promega) close to the manufacturer's instructions with few deviations.

1. We prepare only a quarter of the standard reaction volume for two time points and a master mix of DNA solution (111.11 µg/mL in DNAse-free water).
2. 5 µL of each sample are mixed with 32 µL of 1× SDS-loading buffer and frozen in liquid nitrogen to stop translation and possible degradation.
3. When all samples are taken, they are heated at 65 °C for 15–30 min to avoid protein degradation during storage.

4. The entire sample is carefully loaded to the SDS-PAGE for western blotting. Clotting of the reticulocyte lysate makes a complete sample transfer to the SDS-PAGE very difficult, however, that can be avoided by following this procedure.

3.8 Protein Expression in Bacteria

To define a specific N-terminal of a recombinant protein, we express them as a chimeric fusion with an N-terminal $(His)_8$-MBP-tag followed by a TEV-cleavage site as outlined in detail above and in Figs. 3 and 4. The fusion protein is purified in two steps, first, via

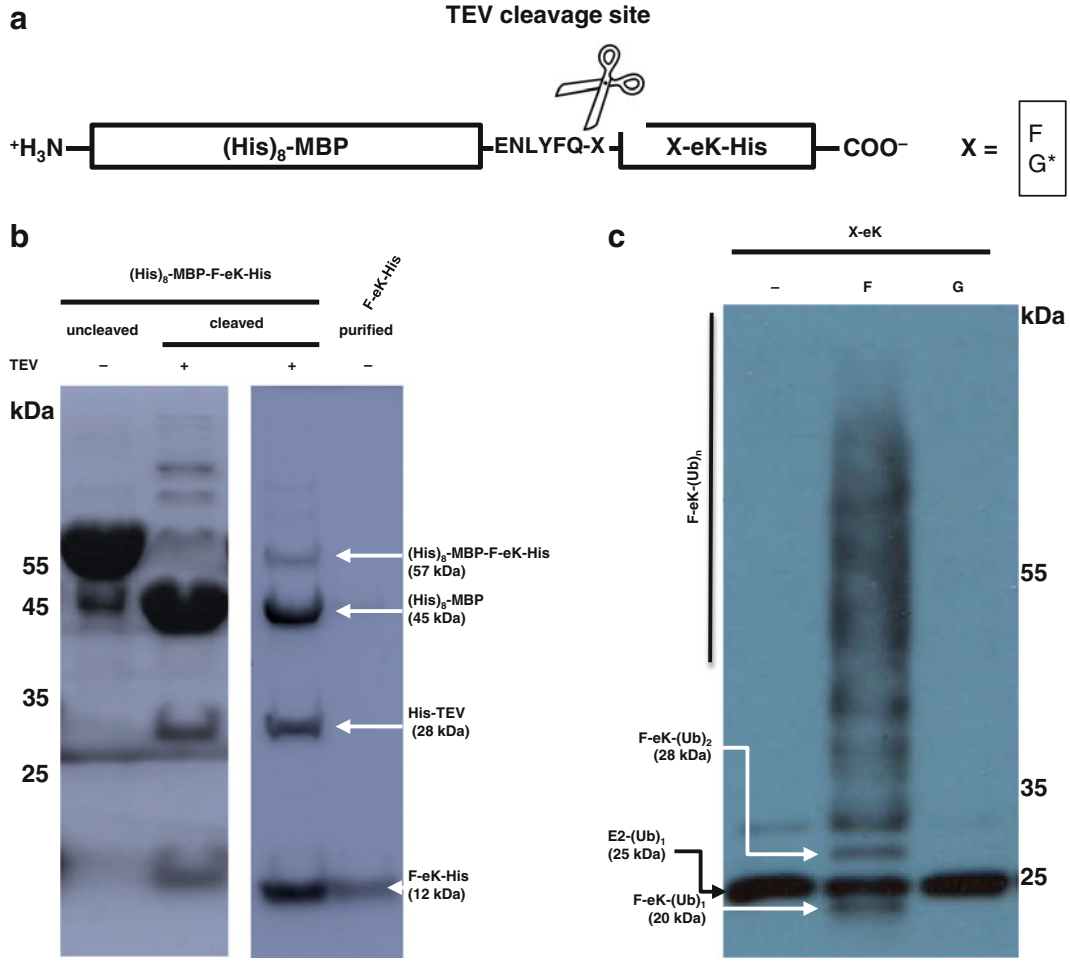

Fig. 4 TEV cleavage and ubiquitination of artificial NERD substrates. (**a**) Fusion protein used to generate desired N-termini at the eK-His artificial substrate using TEV protease. (**b**) Western blot (α-His, sc-803) after SDS-PAGE monitoring the efficiency of the TEV cleavage of the $(His)_8$-MBP-F-eK-His fusion protein and purified artificial substrate F-eK, (1) purified fusion protein, (2) and (3) reaction mixture after TEV cleavage of the fusion protein, and (4) final F-eK-His substrate after further purification first via amylose resin, second via Ni-NTA agarose, then via centrifugal filter units to remove $(His)_8$-MBP, uncleaved fusion protein and the His-tagged TEV protease. (**c**) In vitro ubiquitination assay of the purified F-eK, with G-eK as negative control on an anti-Ub western blot (α-Ub, sc-8017). *: not per se destabilizing

His tag directly followed by purification using the MBP tag. The expression volume is dependent on the amount of protein which is needed. We usually use 200 mL of cultures in 500 mL Erlenmeyer flasks. The expression vectors pVP16-tev-POI are transformed into *E. coli* BL21-CodonPlus(DE3)-RIL.

1. 4 mL of overnight preculture are prepared and 1 mL added to the main expression culture containing 200 mL of LB medium (1:200 inoculation) supplemented with appropriate antibiotics, in our case Carbenicillin at 50 μg/mL.
2. Grow the culture until OD_{600} of 0.3 (*see* **Note** 7).
3. Induce the expression by adding 200 μL of 1 M IPTG to 200 mL of main culture, shift the cultures to 20 °C and express for 16–18 h.
4. Harvest the bacteria by centrifugation (3500 × *g*, 4 °C, 10 min) and resuspend the pellet in 10 mL of Ni-buffer. The pellet can be stored at −20 °C for months.
5. To break down the cell walls, add 250 μL of lysozyme solution for 1 h on ice followed by cell disruption by French press. Immediately add 100 μL of 100 mM PMSF (for 1 mM PMSF final concentration) to inhibit proteases.
6. Centrifuge the lysate at 18,500 × *g* for 25 min, load the clear supernatant to the Ni-NTA agarose (nickel-charged resin) column of 1 mL bed volume equilibrated with Ni-buffer. In parallel, equilibrate an amylose column (1 mL bed volume) with amylose buffer for the second purification step. To get a better binding to the column, we recommend letting the supernatant flow through the column for three times.
7. Wash the column with 15 mL of Ni-buffer.
8. Equilibrated the amylose column with amylose buffer.
9. Elute the proteins with 5 mL of Ni-elution buffer containing 200 mM imidaziole and directly load the eluate to the amylose column which was previously equilibrated in the same buffer. Again, to achieve better binding let it pass three times over the column.
10. Wash column with 15 mL of amylose buffer.
11. Elute proteins in five fractions of 500 μL with amylose-elution buffer containing 10 mM maltose.
12. To prove that the protein was purified properly, we recommend taking samples from each step and monitoring the purity via SDS-PAGE or western blot, both procedures are described below.
13. The material is ready for TEV-mediated digestion and purification.

3.9 TEV Cleavage

1. Determine the OD_{280} of the eluate of the amylose column and of the TEV stock solution (*see* **Note 8**).
2. Mix fusion protein solution with TEV protease solution in a 100:1–50:1 ratio of OD_{280} and incubate for 16–18 h at 4 °C.
3. The reaction mix is loaded on centrifugal filter units to replace amylose-buffer containing maltose by amylose buffer lacking with maltose. The reaction mix is reduced by centrifugation to a volume of 1 mL and then diluted into 15–20 mL of amylose buffer without maltose. The reaction mixture can further be dialyzed against 3 L of amylose buffer for 6–16 h to remove the maltose.
4. Load the mix to an amylose column equilibrated with amylose buffer, here again to increase binding of MBP tag and uncleaved fusion protein, let it pass the column three times.
5. Harvest the flow through and elute the $(His)_8$-MBP tag and uncleaved fusion protein with amylose-elution buffer containing 10 mM maltose to clear the column and re-equilibrated.
6. Repeat the procedure three times to get a pure X-POI.
7. The flow through can be purified further by using a Ni-NTA column again, then also the His-tagged TEV protease is eliminated from the protein mix together with desired X-POI, in our case F/G-eK-His (Fig. 4a, b).
8. Due to the size difference, we recommend to use centrifugal filter units to separate and concentrate the proteins (*see* **Note 9**). Using 30 kDa cut-off centrifugal filters will retain the TEV protease but allow the F-eK-His to pass-through and this can be finally concentrated using 10 kDa cut-off centrifugal filters (*see* **Note 10**).
9. Check protein concentration and purity on SDS-PAGE and western blot.

3.10 Protein Concentration Determination

Use your favorite method to determine the overall protein content of the cleared supernatant and follow the manufacturer's instructions. A decent BSA calibration curve is received when a protein standard such as BSA (stock is 1 mg/mL) is added in steps of 0, 2, 4, 6, 8, 10, 12, 16, 20, 24 µL + 2 µL extraction buffer to each tube. The concentrations usually represent the concentrations present in the supernatants to be tested. The linear range of the assay for BSA is 0.2–0.9 mg/mL.

1. Prepare as many cuvettes as needed with protein assay components (800 µL of water, 200 µL of protein assay solution).
2. Add 2 µL of each sample to the sample cuvettes.
3. Invert cuvettes wearing gloves. Make sure not to carry-over any protein from one assay to the other by wiping the gloves with paper towels.

4. Incubate at room temperature for at least 5 min. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 h.
5. Measure the absorbance at 595 nm.
6. Plot the standard and the sample values to extrapolate the protein concentrations.

3.11 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

We recommend the use of mini gels (e.g., from Bio-Rad) for optimal separation of the protein at a thickness of 1.5 mm, the gel is also relatively robust for the subsequent handling.

1. Place the fully assembled SDS-PAGE gel apparatus onto the bench and rinse carefully all the gel pockets that should be used. It helps to run some 2× sample buffer on top of the pockets to visualize where improperly polymerized or clogging polyacrylamide is remaining.
2. Load the molecular weight marker in one pocket. Also load those wells that remain empty and are directly adjacent to the sample wells with SDS-PAGE sample buffer. We use 200-µL gel-loading tips with extended length to load the samples onto a gel. This helps to avoid spill-over.
3. Load the samples carefully into the gel.
4. Run the gel according to the manufacturer's instructions. We set the power supply initially to 85 V and after 15 min increase the voltage to 135 V for 1 h.
5. Switch off the power supply and disassemble the apparatus.
6. Cut between the stacking and the separating gel to remove and discard the stacking gel and cut the remaining part vertically if not all the pockets of the gel have been loaded to remove and discard the empty lanes. Also cut off the dye front and dispose it.
7. Rinse the gel briefly with semi-dry buffer.

3.12 Western Transfer of Proteins and Detection

In the following part, the proteins are blotted to PVDF, probed with the antibodies and detected via chemoluminescence. This allows detection of proteins in extracts of protoplasts, cell-free systems and after TEV cleavage.

1. For each blot, prepare the required number of sheets of Whatman filter paper (five sheets of 0.34 mm thick blotting paper (Whatman 3MM Chr), three sheets of 0.92 mm thick paper (Whatman 17Chr), or two sheets of 1.2 mm thick paper (Whatman GB005)) and one sheet of PVDF membrane of the appropriate size, slightly larger than the gel.
2. Activate the membrane for 2–5 min in methanol.
3. In a clean, fat-free tray, allow the cut gels and filter papers to wet by capillary action and equilibrate the membrane.

4. Assemble the lower part of the blotting sandwich: start with the bottom of gel cassette that will face the anode, filter papers, membrane, gel, filter paper.
5. Make sure to keep all the layers moist and take precautions not to include air bubbles in the setup. Roll out the air in all the layers using a rinsed test tube or glass rod.
6. Activate the power supply and transfer at roughly 1 mA/cm^2 gel size (height × width × 0.65 = mA/gel) for 1–2 h.
7. Once the transfer is complete, open blotting machine, carefully take the membrane into 10 mL of blocking solution (e.g., 5 % (w/v) dry milk in TBST) for 1 h at room temperature or overnight at 4 °C on a rocking platform.
8. Discard the buffer and rinse the membrane quickly in TBST prior to addition of the primary antibody (Table 4).
9. Incubate with primary antibody for 1 h at room temperature while rocking.
10. Remove the primary antibody and wash the membrane three times for 5 min each with 20 mL of TBST.
11. Freshly prepare the secondary horseradish-conjugated antibody (Table 4) and add to the membrane for 1 h at room temperature on a rocking platform.
12. Discard the secondary antibody and wash the membrane three times for 10 min each with TBST.
13. After the final wash, briefly dry the membrane on filter paper and label properly if not done previously.
14. Line the X-ray film cassette with Saran wrap or a plastic bag and position the blot into a wrap or plastic pocket to separate the membrane to be soaked in ECL detection reagent from the film.
15. Mix the ECL reagents according to the instructions, here in a ratio of 1:1, and immediately spread it over the membrane. Ensure even coverage.
16. Squeeze out excess liquid, blot with tissue paper and move towards the dark room with safe light conditions.
17. Expose the first film for a suitable exposure time, typically 30 s, and determine optimal exposure time later on.

4 Notes

1. TEV works here because other proteases such as thrombin and PreScission are characterized by an invariant P1′ residue and therefore may only be used to generate a small set of predefined freshly formed N-termini: thrombin cleaves preferentially

between Arg and Gly of LVPR|GS, PreScission protease between Gln and Gly of LEVLFQ|GP. Theoretically, also enterokinase (cleaves preferentially after Lys at DDDDK|X and at other basic residues, but not at the site if followed by Pro) and factor Xa (cleaves preferentially after Arg at IG/DGR|X and at other basic residues, but not at the site if followed by Pro or Arg) should work to generate artificial NERD substrates.

2. Home-made TEV is produced as follows: *E. coli* BL21(DE3)-RIL cells containing pRK793 (Table 2) are grown at 37 °C in LB containing 100 μg/mM ampicillin or 50 μg/mM carbenicillin (for pRK793) and 30 μg/mL chloramphenicol (for pRIL of the *E. coli* strain). When the cells reach mid log phase (OD_{600} of approx. 0.5), IPTG is added to a final concentration of 1 mM and the temperature is reduced to 30 °C. After 4 h of induction, the cells are collected by centrifugation. The protocol for TEV purification is as follows [53]: Dissolve the cell pellet in 10 mL of lysis buffer (50 mM NaH_2PO_4 (pH 8.0), 100 mM NaCl, 10% (v/v) glycerol, 25 mM imidazole) per 1 g of wet cell paste. Lyse the cells with a Manton-Gaulin homogenizer at 10,000–10,500 psi for three passes. 5% polyethelene imine (adjusted to pH 7.9 with HCl) are added to a final concentration of 0.1%, mixed by inversion and immediately centrifuged at 15,000 g for 30 min. The supernatant is run through a Ni-NTA column equilibrated with lysis buffer, using approx. 2 mL of resin per gram of wet cell paste. The column is washed with 7 volumes of lysis buffer and the TEV protease eluted with a linear gradient of lysis buffer to elution buffer (50 mM NaH_2PO_4 (pH 8.0), 100 mM NaCl, 10% (v/v) glycerol, 200 mM imidazole) in 10 column volumes overall. The appropriate fractions (tested by SDS-PAGE for presence of TEV) are pooled and EDTA and DTT added to a final concentration of 1 mM each. The TEV is concentrated using Amicon Ultra-15 (Merck Millipore) with 20 kDa cut-off. Concentrate the protease to approx. 1 mg/mL and flash freeze in liquid nitrogen. Store at −80 °C and test aliquots for efficiency before using.
3. Acrylamide is a neurotoxin and carcinogenic in the unpolymerized form. Even the polymerized gel contains still traces of it and thus, it has to be handled very carefully and spills and contamination avoided. Any waste has to be disposed off accordingly.
4. TEMED is best stored at room temperature in a desiccator. Buy small bottles as it may decline in quality after opening and thus, gels will take longer to polymerize.
5. To prevent bubbles at the border of the separating gel, it is overlaid with either isopropanol or water-saturated isobutanol. For the latter, shake equal volumes of water and isobutanol in

a glass bottle and allow separation. Use the top layer. Store at room temperature.

6. Calculation of required MMG volume: $V=(x\times10^4\times2)/(2\times10^5)=x/10$ with x=average of number of protoplasts counted in four squares.
7. Consider that different strains behave different in growth speed.
8. The TEV protease catalytic domain is expressed from pRK793 (Table 2) with an N-terminal His-tag and a C-terminal polyarginine tag. The His-tag can be used to eliminate TEV from X-POI after cleavage.
9. Due to its apparent molecular volume and protein shape, TEV does not pass through 30 kDa cut-off centrifugal filter units, even though its size is 28 kDa. Thus, filter units of 10 kDa should be considered.
10. Amicon Ultra 15 centrifugal filters have a maximal loading volume of 15 mL.
11. The pVP16 Gateway bacterial (*E. coli*) expression vector was designed as a $(His)_8$-MBP fusion tag system to overcome the low solubility of recombinant eukaryotic proteins and to provide a generic Ni-IMAC purification strategy. The backbone is derived from pQE80 (Qiagen) to express an N-terminal fusion protein consisting of $(His)_8$-MBP and a linker region containing the TEV protease recognition site contiguous with the first residue of the target protein. TEV protease cleavage site in pVP16 is "partial" and not functional. Therefore, the full TEV cleavage site must be added directly 5′ to the first codon of the insert by PCR, which allows amino acids encoded by the attB1 site to be cut off after protein expression.

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