

Proteolytic degradation of the flavonoid regulators, TRANSPARENT TESTA8 and TRANSPARENT TESTA GLABRA1, in *Arabidopsis* is mediated by the ubiquitin/26S proteasome system

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Regulated proteolysis by the ubiquitin/26S proteasome system (UPS) has emerged as a major posttranslational control mechanism regulating transcription factor (TF) activity in plants. Anthocyanin biosynthesis in *Arabidopsis* is regulated by a ternary complex comprised of basic helix-loop-helix (bHLH), R2R3MYB and WD-repeat (WDR) proteins. The bHLH TF, TRANSPARENT TESTA 8 (TT8), and the WDR protein, TRANSPARENT TESTA GLABRA1 (TTG1), are essential for expression of late flavonoid biosynthesis genes. Previous studies have demonstrated that the turnover of several anthocyanin pathway regulators is controlled by the UPS. Here, we show that TT8 and TTG1 are short-lived and targeted by the UPS for degradation. Our findings further extend our understanding of the role of the UPS in the regulation of anthocyanin biosynthesis in plants.

The anthocyanin biosynthesis pathway is one of the most extensively studied metabolic pathways in plants. Biosynthesis and accumulation of anthocyanins is dependent upon several factors including light, phytohormones and nutrients.¹⁻³ Upon perception of developmental or environmental signals, regulatory proteins begin accumulating in the cell, and subsequently, trigger the expression of structural genes leading to biosynthesis of anthocyanins. In plants studied to date, a ternary transcription factor (TF) complex, comprised of basic helix-loop-helix (bHLH), R2R3MYBs and WD-repeat (WDR) proteins, regulates the transcription of structural genes in the pathway.^{4,5} Recent studies suggest that, in addition to transcriptional control, the anthocyanin pathway is also regulated by posttranscriptional and posttranslational control mechanisms.⁶⁻¹⁰ To maintain cellular homeostasis, cells control the transcriptional machinery either through the expression of negative regulators, small RNAs, or by selectively degrading the proteins involved in metabolic pathways. In *Arabidopsis*, the single repeat MYBs, CAPRICE (CPC) and MYBL2, act as negative regulators of the anthocyanin biosynthesis pathway.¹¹⁻¹³ They compete with the R2R3MYBs, PAP1/PAP2, to interact with bHLH factors to form an inactive/repressor complex thereby affecting the transcription of structural genes. The small RNAs have also been shown to play a

critical role in the anthocyanin pathway by regulating expression of the regulators such as PAP1.^{6,8}

Both TFs and enzymatic proteins have an intrinsic half-life depending on their function and cellular requirement. Metabolic enzymes are generally more stable than TFs and the rapid turnover of TFs is considered to be a part of the regulatory mechanism.¹⁴ The ubiquitin/26S proteasome system (UPS) has emerged as the most prevalent posttranslational control mechanism dictating the activities of TFs in plants. One hypothesis for the rapid turnover of regulatory proteins is that timely degradation of the “spent” regulatory proteins allows promoter clearance for new rounds of transcription.¹⁵ In *Arabidopsis*, loss of 26S proteasome function results in higher accumulation of anthocyanin pigments in vegetative tissues indicating the involvement of UPS in anthocyanin regulation.^{16,17} This hypothesis is strengthened by recent reports demonstrating that the activities of TFs involved in the regulation of anthocyanin biosynthesis in plants are regulated by UPS. The R2R3MYBs, PAP1 and PAP2 in *Arabidopsis*, and MdMYB1 in apple, are degraded in the dark by the UPS suggesting that the light-mediated anthocyanin accumulation in *Arabidopsis* and apple skin is due, at least in part, to the stabilization of these factors in light.^{7,9} We have recently shown that the bHLH factors, GL3

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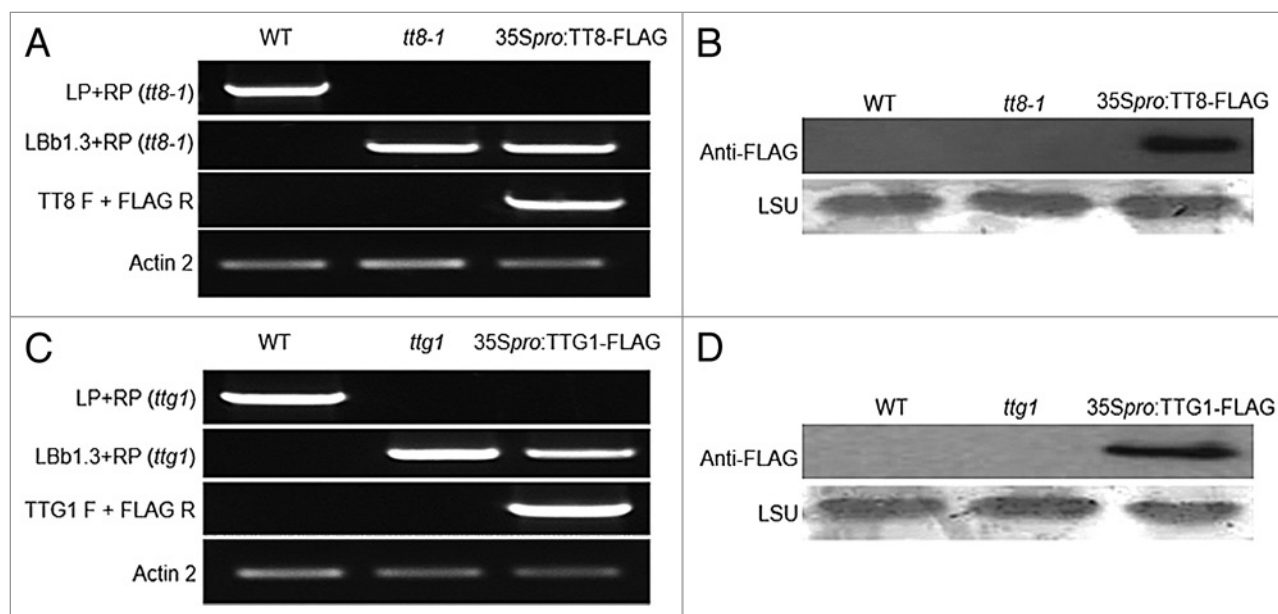


Figure 1. Genotyping and Western blot analysis of homozygous *tt8-1* and *ttg1* plants expressing FLAG-tagged TT8 and TTG1. (A) DNA was isolated from 2-week-old plants. Homozygosity was confirmed by PCR. TT8-FLAG transcripts were PCR amplified from total RNA isolated from 2-week-old seedlings using TT8-F and FLAG-R primers. *Actin 2* was used as a control. (B) Total protein was isolated from 2-week-old seedlings and the presence of TT8-FLAG fusion protein was checked by Western blot using Anti-FLAG M2 antibody. Ponceau S-stained membrane with LSU is shown as a loading control. (C) and (D) Genotyping, RT-PCR, and Western blot analysis were performed for the plants expressing TTG1-FLAG as in (A) and (B).

and EGL3 that control anthocyanin biosynthesis and epidermal cell differentiation in *Arabidopsis*, are short-lived and targeted by the UPS for degradation.¹⁰

In *Arabidopsis*, the bHLH TF TT8 serves as a component of the regulatory complex that positively regulates the expression of most of the late biosynthesis genes in the flavonoid pathway leading to the accumulation of anthocyanins and proanthocyanidins.¹⁸ The WD-repeat protein TTG1 works coordinately with the bHLH and R2R3MYB TFs to regulate flavonoid biosynthesis and trichome development in *Arabidopsis*.¹⁹ Here, we demonstrate that TT8 and TTG1 are also short-lived and are targeted by UPS for proteolytic degradation.

To test the stability of TT8 and TTG1 we ectopically expressed both TFs in stable transgenic lines in their respective homozygous *tt8-1* (SALK_030966) and *ttg1* (Salk_104152) mutant backgrounds. Homozygosity of the mutants was confirmed by PCR (Fig. 1A and C). Modified pCambia1300 vector carrying full-length cDNA of *TT8* or *TTG1* were used for plant transformation. For efficient immunodetection, both TFs were C-terminally fused to a 3'FLAG tag. Homozygous transgenic plants were verified for accumulation of the fusion proteins by Western blot (Fig. 1B and D). Ectopic expression of *TT8* or *TTG1* did not show any adverse effects on the overall growth and development of homozygous transgenic lines.

We then independently investigated whether TT8 and TTG1 are targeted for degraded by UPS. Two-week-old seedlings of 35Spro:TT8-FLAG or 35Spro:TTG1-FLAG transgenic plants were treated with 200 μ M cycloheximide (CHX) in half-strength MS medium at room temperature for different time intervals and the depletion rates of TT8-FLAG and TTG1-FLAG proteins

were monitored by Western blot analysis using anti-FLAG-M2 monoclonal antibody (Sigma). The amount of TT8 or TTG1 fusion protein was depleted significantly after 120 minutes of incubation of the seedlings in CHX (Fig. 2A and C). The transcript levels of TT8-FLAG/TTG1-FLAG remained unchanged after CHX treatment for 60 and 120 minutes, indicating that the depletion of the TT8 and TTG1 is posttranslational and the TF proteins are unstable and degraded in the plant cell (Fig. 3A and C). Next, in order to test whether degradation of TT8 and TTG1 is dependent on 26S proteasome, 2-week-old seedlings of the transgenic lines were treated with either CHX alone or in combination with MG132, an inhibitor of proteasome activity, for 120 minutes. DMSO-only treated plants served as control. Degradation of both TT8 and TTG1 proteins were inhibited significantly by MG132 (Fig. 2B and D). Similar to CHX treatment, the combined CHX and MG132 treatment did not affect the transgene transcriptional level (Fig. 3B and D). Taken together, our results demonstrate that, in *Arabidopsis*, TT8 and TTG1 proteins are short-lived and targeted for proteasomal degradation.

Transcriptional regulation is considered to be the major mechanism dictating metabolic pathway gene expression in plants. However, recent studies on the regulation of flavonoid biosynthesis pathways suggest that posttranscriptional and post-translational mechanisms also play significant roles in the regulation of metabolic pathways.⁶⁻¹⁰ The UPS has been demonstrated to regulate the activities of key regulatory proteins, including GL3, EGL3, PAP1 and PAP2, in the *Arabidopsis* flavonoid pathway.^{9,10} Here we show that two additional TFs, TT8 and TTG1, in the pathway are also targeted for proteasomal degradation,

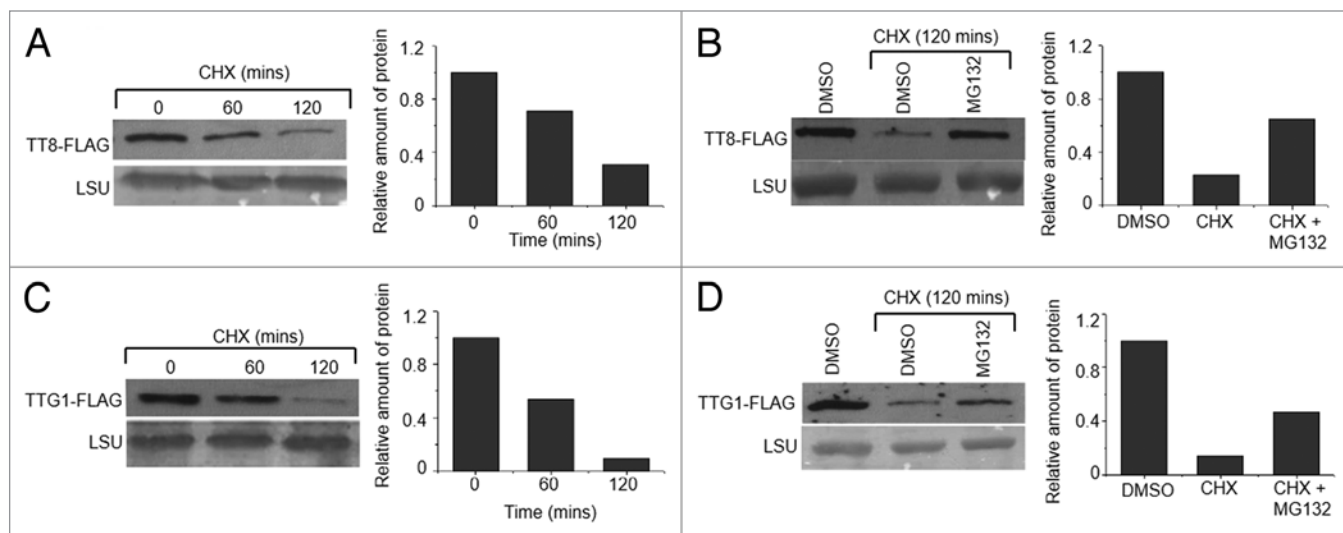


Figure 2. (A) Stability assay for TT8. Two-week-old *tt8-1* seedlings expressing a 35Spro:TT8-FLAG (TT8-FLAGox) transgene were treated with 200 μ M cycloheximide (CHX) for the indicated time period and used for Western blot analyses with anti-FLAG antibodies. Ponceau S-stained membrane with LSU is shown as a loading control. The relative amount of protein in each sample is normalized against the corresponding LSU. (B) Stabilization of TT8 by MG132. Two-week-old *tt8-1* seedlings expressing a 35Spro:TT8-FLAG were treated with CHX, alone or in combination with MG132 (200 μ M), for 120 minutes and used for Western blot analyses with anti-FLAG antibodies. DMSO-only treated plants served as control. The relative amount of protein in each sample is normalized against the corresponding LSU. (C) and (D) TTG1 stability and MG132 stabilization assays were performed using two week-old *ttg1* plants expressing a 35Spro:TTG1-FLAG (TTG1-FLAGox) as in (A) and (B).

suggesting that the activities of most, if not all, TFs in the ternary complex that regulate the flavonoid pathway in *Arabidopsis* are regulated by UPS. UPS mediated degradation is a multistep process that involves several enzymatic reactions. E3 ligases interact with target proteins to facilitate their degradation through UPS. Proteasomal degradation of PAP1 and PAP2 is mediated by the E3 ligase, COP1/SPA.⁹ We recently demonstrated that the HECT domain-containing E3 ligase, Ubiquitin Protein Ligase 3 (UPL3), interacts with GL3 and EGL3 and mediates their degradation through the UPS.¹⁰ The E3 ligase protein that mediates the degradation of TT8 and TTG1 has yet to be identified. Collectively, these findings highlight the importance of UPS in flavonoid pathway regulation in plants.

Materials and Methods

Plant materials and growth conditions

All mutant and transgenic lines were in *Arabidopsis thaliana* ecotype Col0. The TDNA insertion mutant lines, *tt8-1* (SALK_030966) and *ttg1* (Salk_104152), were obtained from The Arabidopsis Biological Resource Center (ABRC). For germination, seeds were sterilized using 75% ethanol and 30% commercial bleach, and plated on half-strength MS medium (Caisson Labs). Seeds were stratified for 2 days, and plants were grown in a controlled environment chamber (16-h-light at 22–24 °C and 8-h-dark at 17–19 °C). DNA was isolated from 2-week-old plants. Homozygosity was confirmed by PCR using gene specific LP and

RP primers, as well as by using LBb1.3 and gene specific RP primers.

To generate *TT8* and *TTG1* overexpression lines, full-length cDNAs were cloned into a modified pCambia1300 vector containing the *CaMV35S* promoter and *rbcS* terminator. Three tandem repeats encoding the FLAG epitope (3'GACTACAAAG ACGATGACGA CAAA) were fused inframe to the C-terminal end of *TT8* and *TTG1* cDNA. The resulting constructs, 35Spro:TT8-FLAG or 35Spro:TTG1-FLAG were introduced into *Agrobacterium tumefaciens* strain GV3850

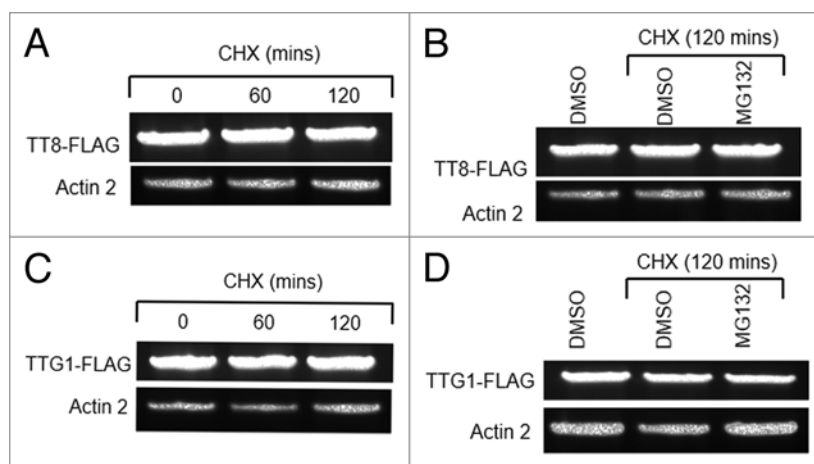


Figure 3. TT8-FLAG transcript levels in the seedlings, either untreated or treated with 200 μ M CHX, were examined using RT-PCR. *Actin 2* was used as a control. TT8-FLAG transcript levels in the seedlings, treated with either DMSO or 200 μ M CHX (\pm 200 μ M MG132) for 120 minutes, were detected using RT-PCR. *Actin 2* was used as a control. (A) and (D) TTG1 transcript levels were detected in 2 week-old *ttg1* plants expressing 35Spro:TTG1-FLAG (TTG1-FLAGox) as in (A) and (B).

and used for transformation of *tt8-1* and *ttg1* plants by the floral dip method.²⁰ To check for the TT8-FLAG or TTG1-FLAG transcripts, total RNA was isolated from 2-week-old seedlings and used for first strand cDNA synthesis. TT8-FLAG or TTG1-FLAG transcripts were PCR amplified using forward primer TT8-F (5'-ATGGATGAAT CAAGTATTAT TCC-3') or TTG1-F (5'-ATGGATAATT CAGCTCCAGA TT-3') in combination with reverse primer FLAG-R (5'-TTTGTCTGTCATCGCTTTTGT AGTC-3'). *Actin 2* was amplified using Actin 2 F (5'-AACCCAAAGG CCAACAGAGA-3') and Actin 2 R (5'-AAGGTCACGT CCAGCAAGGT-3') primers and used as experimental control.

Treatments

For protein stability and proteasome inhibition assays, 2-week-old seedlings were incubated with either 200 μ M cycloheximide (CHX) alone or in combination with 200 μ M MG132. The CHX and MG132 stocks were prepared in water and DMSO, respectively, and all control experiments contained an equal amount of solvent.

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Immunoblotting analysis

Total protein extracts were prepared as described previously,²¹ separated by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). Membranes were stained with Ponceau S to ensure equal loading, blocked with 3% BSA, probed with the anti-FLAG antibody (clone M2, Sigma) followed by the HRP-conjugated secondary antibody (Thermo Scientific). Signal was detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). The amount of proteins present relative to the untreated or only solvent treated plants were determined densitometrically using ImageJ (<http://rsb.info.nih.gov/ij/>) software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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