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Report

Export of FT Protein from Phloem Companion Cells Is Sufficient for Floral Induction in *Arabidopsis*

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Summary

Several endogenous and environmental factors need to be integrated to time the onset of flowering [1–3]. Genetic and molecular analyses, primarily in *Arabidopsis thaliana* and rice, have shown that *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) play central roles in photoperiod-dependent flowering [4–13]. The overall picture is that CO acts in the phloem companion cells of leaves and that its main effect is to induce *FT* mRNA in these cells [11, 12, 14–19]. Surprisingly, FT, a small globular protein of 20 kDa, interacts at the shoot apex with the bZIP transcription factor FLOWERING LOCUS D (FD) to induce downstream targets [17, 18]. Given that green fluorescent protein (GFP), which as a monomer is 27 kDa, can be easily exported to sink tissue including flowers when expressed in phloem companion cells, the latter finding strongly implied that FT protein is the mobile floral-inductive signal [17–19]. In agreement with this hypothesis, an FT-GFP fusion, just like GFP, can be exported from the phloem of both rice and *Arabidopsis* [20, 21]. It has been unknown, however, whether mobile FT protein is sufficient for transmitting the flowering signal. Here we show that *FT* mRNA is required in phloem companion cells where it acts partially redundant with its paralog *TWIN SISTER OF FT* (*TSF*) to induce flowering. Furthermore, we have devised a method that uncouples *FT* mRNA and protein effects in vivo. We demonstrate that export of FT protein from phloem companion cells is sufficient to induce flowering.

Results and Discussion

FT mRNA Is Required in Phloem Companion Cells

It has been shown that ectopic expression of *FT* mRNA at the shoot apex proper, which includes the shoot meristem and young primordia, is sufficient to induce flowering, consistent with FT protein acting at the shoot apex through its direct interaction with the shoot-apex-specific FD protein [16–18]. To address whether *FT* mRNA is not only sufficient but also necessary at the shoot apex, we made use of artificial microRNAs (amiRNAs) [22]. Like endogenous miRNAs, amiRNAs can target mRNAs with sequence complementarity for specific degradation and thus provide a powerful tool for tissue-specific mRNA inactivation. Expression of an amiRNA against the *FT* mRNA (*amiR-FT*) from the constitutive *35S* promoter delays flowering to the same extent as complete inactivation of the *FT* gene by genetic means [22]. We observed a similar delay in flowering when we expressed *amiR-FT* under control of the *SUC2* promoter, which is specific for phloem companion cells [23, 24]. This indicates that expression of *FT* mRNA in companion cells is not only sufficient for the induction of flowering, but that *FT* mRNA expression in companion cells is required for *FT* function (Table 1 and Figure 1) [16]. In contrast, targeted destruction of *FT* mRNA in cells that express the FT interactor *FD*, by expression of *amiR-FT* from the *FD* promoter, had no effect on flowering time. This observation suggests that *FT* mRNA is not required in the *FD* expression domain. It also indicates that the effect of *SUC2::amiR-FT* on flowering is not a consequence of export of the amiRNA from the phloem to the shoot apex.

TSF mRNA Acts Redundantly with *FT*

Because *FT* acts partially redundant with a close paralog, *TWIN SISTER OF FT (TSF)* [25, 26], we repeated the amiRNA experiment with an amiRNA that simultaneously targets both *FT* and *TSF*. Constitutive expression of *amiR-FT/TSF* phenocopied the extremely late flowering of the *ft tsf* double mutant (Figure 1 and Table 1). Similar to *amiR-FT*, *amiR-FT/TSF* delayed floral transition efficiently when expressed in the phloem, but not at the apex. Thus, there does not appear to be a separation in spatial requirement for *FT* and *TSF* function, and neither mRNA appears to be required in the *FD* expression domain at the shoot apex. Our observation that *FT* mRNA does not appear to be required in the cells where the protein is active indicated that FT protein is at least part of the mobile, *FT*-dependent signal. In this context, it is important to note that a study that had proposed *FT* mRNA to act as a transmissible signal has been retracted [27].

It has recently been shown that the homeodomain protein FWA can inactivate the FT protein and that expression of FWA from the FD promoter is sufficient to delay flowering [28] (Table S1 in the Supplemental Data available with this article online). These findings indicate that the *FD* promoter is functional, further strengthening the idea that FT protein is required at the apex and is likely to be transported from the vasculature to the apex.

TEV Protease Can Be Expressed in *Arabidopsis*

To test whether FT protein movement is also sufficient to induce flowering, we first immobilized FT protein, which is normally found in both the nucleus and the cytoplasm [17], by fusing it to three consecutive copies of yellow fluorescent protein (YFP). The FT:TEV^{rs}:3xYFP:NLS fusion protein is more than 112 kDa, which is more than five times the molecular weight of native FT. The FT and 3xYFP:NLS portions of FT:TEV^{rs}:3xYFP:NLS were

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Table 1. Flowering Time of Transgenic Plants, Expressed as Leaves in the Main Shoot

Genotype	Leaves	Deviation	Range	n
Experiment 1				
Col-0 (wild-type)	16.1	±0.8	14–20	20
ft-10	41.0	±1.3	35–47	20
ft-10 tsf-1	59.8	±1.7	54–66	20
35S::FT	7.6	±0.5	6–9	19
FD::FT	7.0	±0.4	5–9	20
SUC2::FT	5.4	±0.3	5–7	19
Experiment 2				
Col-0 (wild-type)	14.0	±0.8	11–19	20
35S::amiR-FT (#NW36_1-26)	39.1	±1.4	33–45	20
FD::amiR-FT (#NW39_1-9)	12.4	±0.8	10–17	29
SUC2::amiR-FT (#NW48_1-1)	41.8	±1.9	32–48	20

Flowering time is shown for the T2 progeny of one T1 line per transgene. Deviation is given as 2× standard error of mean (SEM). Flowering time of the parental T1 lines (*35S::amiR-FT, #NW36_1-26; FD::amiR-FT, #NW39_1-9; SUC2::amiR-FT, #NW48_1-1*) is indicated in Figure 1 (arrows). All transgenic lines are in Col-0 background. Flowering time for additional, independent *amiR-FT* T2 lines is shown in Table S1.

separated by a recognition site for the Tobacco Etch Virus protease (TEV protease), which we hoped would allow us to release mature FT protein from the fusion protein (Figure 2). TEV protease has high substrate specificity [29], and no annotated endogenous *Arabidopsis* protein contains a canonical TEVP recognition site (ENLYFQ|G). Consistent with TEV protease being harmless, plants expressing the gene from either the constitutive *35S* promoter or the companion-cell-specific *SUC2* promoter were healthy and undistinguishable from wild-type. Most importantly, TEV protease had no effect on flowering time (Table 2 and Figures 3C and 3D). Expression of *TEV* protease mRNA in these plants was confirmed by RT-PCR (Figure S1).

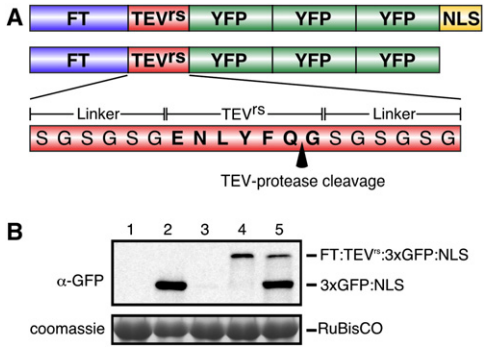

Figure 2. Proteolytic Release of FT Protein from an Immobile Precursor

(A) Diagrams of FT:TEV^{rs}:3x-YFP fusion proteins with and without nuclear localization signal (NLS). TEV protease recognition site is indicated in bold letters, and the cleavage site is marked with an arrowhead.

(B) Protein blot analysis of plants expressing FT:TEV^{rs}:3xYFP:NLS and TEV protease under the control of a *SUC2* promoter. Lane 1, Col-0 (wild-type control); lane 2, 3xYFP:NLS; lane 3, TEV protease; lane 4, FT:TEV^{rs}:3xYFP:NLS; lane 5, FT:TEV^{rs}:3xYFP:NLS, TEV protease. Coomassie-stained gel, showing the large subunit of RuBisCO as loading control.

FT:TEV^{rs}:3xYFP:NLS mRNA and Protein Is Trapped in Phloem Companion Cells

Expression of *FT:TEV^{rs}:3xYFP:NLS* from both the constitutive *35S*- or the apex-specific *FD*-promoter induced flowering in a manner similar to transgenic expression of

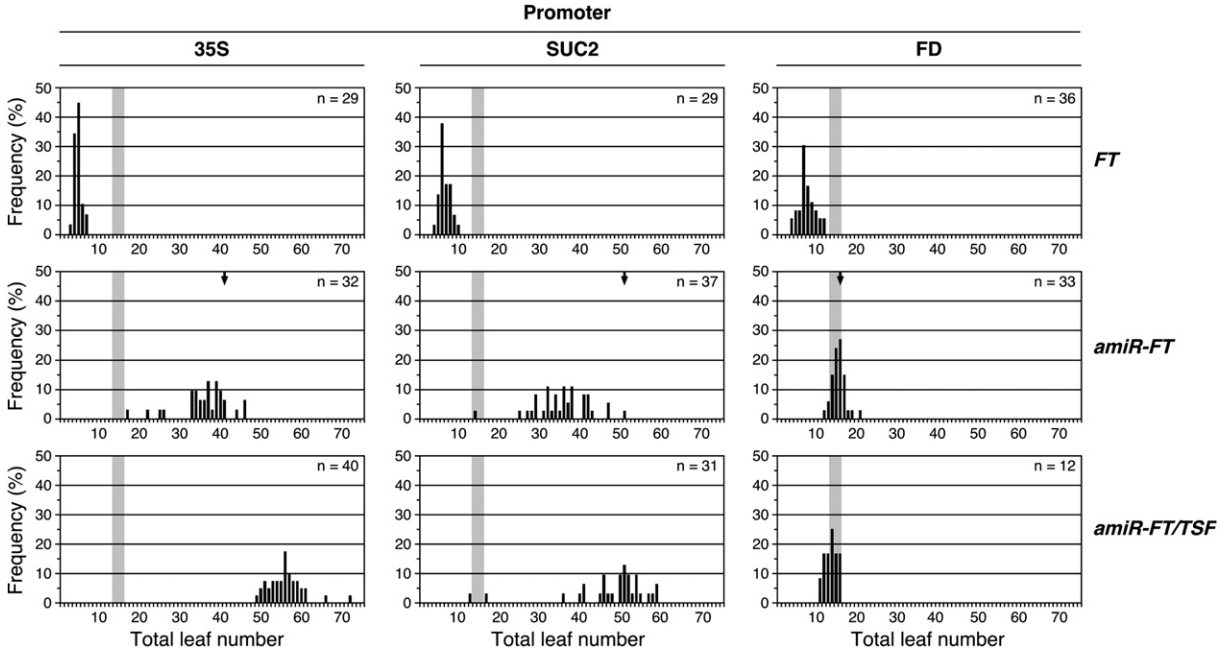

Figure 1. Effect of Artificial MicroRNAs against *FT* and *TSF* mRNA on Flowering Time in *Arabidopsis thaliana*

Histograms of flowering time of independent T1 lines are shown. Number (n) of independent T1 lines analyzed per transgene is displayed in each panel. Flowering time is given as leaves on the main shoot. Flowering time of *amiR-FT* lines analyzed in subsequent generations (Table 1) is marked (arrows). Flowering time of Col-0 control plants is indicated (gray box).

Table 2. Flowering Times of Plants with Immobilized/Released FT Protein

Genotype	Leaves	Deviation	Range	n
Col-0 wild-type	12.7	±0.5	10–16	40
35S::TEVP (#JM158-1)	11.7	±0.3	9–14	40
SUC2::TEVP (#JM167-2)	12.7	±0.3	11–15	39
SUC2::3xYFP:NLS (#JM164-5)	15.8	±0.6	11–18	29
35S::FT:TEV^{rs}:3xYFP (#JM157-2)	6.1	±0.2	5–7	22
35S::FT:TEV^{rs}:3xYFP:NLS (#JM156-2)	6.1	±0.2	5–7	25
SUC2::FT:TEV^{rs}:3xYFP (#JM166-2)	12.6	±0.4	10–16	37
SUC2::FT:TEV^{rs}:3xYFP:NLS (#JM165-2)	14.6	±0.4	12–17	29
SUC2::TEVP; SUC2::YFP:NLS	14.7	±1.4	11–18	11
SUC2::TEVP; SUC2:FT:TEV^{rs}:3xYFP	6.0	±0.3	4–7	17
SUC2::TEVP; SUC2::FT:TEV^{rs}:3xYFP:NLS	6.1	±0.2	5–7	30

Flowering time is shown for the T2 progeny of one T1 line per transgene and their F1 progeny. Deviation is given as 2× standard error of mean (SEM). Data obtained from independent T2 lines are reported in Table S1. Abbreviations: TEVP, TEV protease; TEV^{rs}, TEV protease recognition site.

unmodified *FT* (Tables 1 and 2 and Figures 3I and 3J). In contrast, expression of *FT:TEV^{rs}:3xYFP:NLS* from the companion-cell-specific *SUC2* promoter [23, 24] was ineffective in promoting flowering (Table 2 and Figures 3E and 3F). Although YFP fluorescence was strong in companion cells, no YFP signal was detectable at the shoot apex, confirming that FT:TEV^{rs}:3xYFP:NLS is immobile (Figure S2). Together, these findings indicate that neither *FT:TEV^{rs}:3xYFP:NLS* mRNA nor the fusion protein are exported from the companion cells to the apex.

Release of FT Protein Is Sufficient for Floral Induction

To test whether FT protein could move from companion cells to the shoot apex, we generated plants that expressed both FT:TEV^{rs}:3xYFP:NLS and TEV protease in companion cells by crossing parental T1 strains that were hemizygous for a single *SUC2::FT:TEVrs:3xYFP:NLS* transgene (#JM165-2) and *SUC2::TEVP* transgene (#JM167-2), respectively. In 50% (58/115) of F1 progeny, we detected YFP fluorescence in the vasculature. Of these 58 plants, 30 (52%), or 26% of all F1 plants, were early flowering, similar to what had been observed in *SUC2::FT* or *35S::FT:TEV^{rs}:3xYFP:NLS* lines (Table 2 and Figures 3G and 3H). Early flowering cosegregated with the expression of both transgenes. The most obvious explanation for this observation is that upon release of the approximately 20 kDa FT protein from the 112 kDa fusion protein by targeted proteolysis, FT moves from phloem companion cells to the shoot apex, where it interacts with FD to induce flowering. Similar results were obtained with a *SUC2::FT:TEVrs:3xYFP* line (#JM166-2), which lacks the nuclear-localization signal (NLS), indicating that nuclear localization is not required to trap the FT fusion protein in the phloem companion cells (Table 2). To confirm accurate proteolytic cleavage of the FT:TEV^{rs}:3xYFP:NLS protein by TEV protease, we performed protein-blot analysis with antibodies against YFP (Figure 2B). In protein extracts of singly transgenic *SUC2::FT:TEV^{rs}:3xYFP:NLS* plants, we detected FT:TEV^{rs}:3xYFP:NLS at its calculated molecular weight of 112 kDa (Figure 2B). In plants coexpressing TEV protease in companion cells, we observed an additional band that corresponds in molecular weight to a 3xYFP:NLS control (90 kDa) (Figure 2B). The measured difference corresponds closely to the molecular

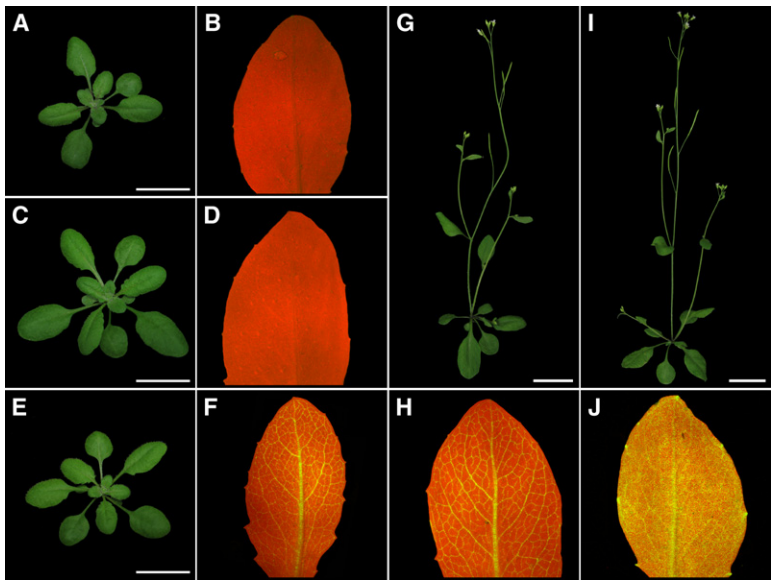

Figure 3. Effect of the Release of FT Protein from Phloem Companion Cells on Flowering

(A–F) Col-0 wild-type (A, B), *SUC2::TEVP* (C, D), and *SUC2::FT:TEV^{rs}:3xYFP:NLS* (E, F) plants grown in long days (16 hr light; 23°C). YFP fluorescence signal (B, D, F) in rosette leaves (only present in [F]).

(G and H) *SUC2::TEVP SUC2::FT:TEV^{rs}:3xYFP:NLS* doubly transgenic plants flower early (G) and express YFP in the vasculature (H).

(I and J) Expression of *FT:TEV^{rs}:3xYFP:NLS* from the constitutive *35S* promoter results in early flowering (I), with YFP detectable throughout the plant (J).

Scale bars represent 1 cm.

weight of 21 kDa calculated for the 175 amino acid FT protein, with 12 additional amino acids of the TEV protease recognition sequence (Figure 2B).

Conclusions

It has been shown before that the *FT* gene is transcribed in phloem companion cells and that *FT* is effective when overexpressed in these cells [15–17]. We have now demonstrated that the presence of endogenous mRNA of *FT* in companion cells is essential for photoperiodic induction of flowering. In addition, the mRNA of *TSF*, a paralog of *FT*, also seems to exert its function in the companion cells.

An *FT:TEV^{rs}:3xYFP:NLS* transgene expressed in companion cells is, however, not sufficient to induce flowering, even though the encoded protein is functional when expressed directly at the shoot apex. The inability of the *SUC2::FT:TEV^{rs}:3xYFP:NLS* transgene to induce flowering indicated that neither the mRNA nor the protein produced by the fusion gene moves from the companion cells to the shoot apex. This finding also lets us rule out that functional FT protein expressed in the companion cells is sufficient for the induction of a downstream signal (including *FT* mRNA expressed from the endogenous locus) that would communicate a floral inductive stimulus from the phloem to the shoot apex. In contrast, release of FT protein from the companion cells is sufficient to induce flowering, suggesting that FT protein is an important component of the mobile florigen signal. Our observations are fully consistent with a report from tomato, where an *FT* ortholog could effectively induce flowering across a graft junction, even though no *FT* RNA movement across the graft junction could be detected [30, 31]. The tomato experiments could not discriminate between two alternative mechanisms, protein trafficking versus initiation of a downstream signal. Our experiments with an immobile FT variant expressed in companion cells argue strongly against such a secondary signal. Our findings are in agreement with a recent report that a 47 kDa FT-GFP fusion protein can move from the phloem to the shoot apex in both *Arabidopsis* and rice [20, 21]. Thus, while our data on their own do not rule out that *FT* mRNA movement plays a role in florigenic signaling within the phloem, we propose that FT protein movement from phloem companion cells though sieve elements to the apex is not only possible but also sufficient to induce flowering.

Experimental Procedures

Plant Material and Culture

The Columbia-0 (Col-0) accession was the wild-type. *ft-10* (GABI_290e08) has been described elsewhere [32]. All plants were grown on soil at 23°C, 16 hr light and 8 hr dark, under a 2:1 mixture of Cool White and Gro-Lux Wide Spectrum fluorescent lights, with a fluence rate of 125–175 μmol m^{-2} s^{-1}. All light bulbs were of the same age. Relative humidity was 65%.

Cloning

All cDNAs were amplified by PCR with Fermentas Pfu polymerase and cloned into Gateway entry vectors by T4 DNA ligase-mediated ligation. The *FT* coding sequence was amplified from a plasmid with the oligonucleotides 5′-atg tct ata aat ata aga gac cc-3′ and 5′-cta aag tct tct tcc tcc gca gcc-3′ to produce the plasmid pJM13. To make the *FT:TEV^{rs}:3xYFP:NLS* fusion construct, the TEV recognition site (TEV^{rs}) was fused to the 3′-end of the *FT* coding sequence by PCR with the sense oligonucleotide 5′-gcg gtc gac atg tct ata aat ata aga gac cc-3′ and the two reverse oligonucleotides 5′-gcc gct tcc aga acc tga acc ctg gaa gta caa gtt ctc tcc aga acc tga tcc aga aag tct tct tcc tcc gca gcc act ctc c-3′ and 5′-gcc gct tcc aga acc tga acc ctg gaa g-3′. The PCR product was introduced into BJ36-3xYFP:NLS [33] as a SalI/SmaI fragment to produce pJM142. To remove the NLS from this construct, pJM142 was partially digested with XbaI and EcoRI, blunted with T4 DNA polymerase, and religated to make pJM143. Both constructs (pJM142, pJM143) were subsequently shuttled into the Gateway entry vector pJLBlue[rev] as SalI/XbaI fragments resulting in the final Gateway entry clones pJM147 and pJM148, respectively.

The TEV protease was amplified from a plasmid (kindly provided by M. Ehrmann) with oligonucleotides 5′-atg ttg ttt aag gga cca cgt gat ta-3′ and 5′-tca gtc acg atg aat tcc cgg cga gt-3′ and cloned into the entry vector pJLSmart[rev] to produce pJM149.

The generation of the amiRNA targeting *FT* has been described (*amiR-ft-1*) [22]. For this study, the construct was digested EcoRI/BamHI and the *amiR-FT* was introduced into the Gateway entry vector pJLBlue[rev] resulting in pNW33.

The *amiR-FT/TSF* (pJM95) was also designed according to the rules described [22]. The oligonucleotides used were: oligonucleotide I, 5′-gat tag atc tca gca tac tcg cgt ctc tct ttt gta ttc c-3′; oligonucleotide II, 5′-gac gcg agt atg ctg aga tct aat caa aga gaa tca atg a-3′; oligonucleotide III, 5′-gac gag agt atg ctg tga tct att cac agg tcg tga tat g-3′; and oligonucleotide IV, 5′-gaa tag atc aca gca tac tct cgt cta cat ata tat tcc t-3′. The PCR product was cloned into pGEM-Teasy (Promega) and transferred into pJLBlue[rev].

For plant transformation, genes of interest were recombined into pGREEN-IIS destination vectors into which the different promoters used in this study (*35S*, *FD*, *SUC2*) had been cloned in front of a modified Gateway recombination cassette. pGREEN-IIS is a derivative of pGREEN-II [34], in which the bacterial kanamycin resistance gene has been replaced by a gene that confers resistance to spectinomycin. Recombinations of genes into these destination vectors were carried out with the Gateway LR clonase II Enzyme mix (Invitrogen).

DNA-modifying enzymes were purchased from Fermentas, if not otherwise indicated. The complete sequences of all constructs used are available on request.

Plant Transformation

Plants were transformed by floral dipping as described [35]. Transgenic plants were selected by watering with BASTA (Bayer) at a dilution of 1/1000.

Extraction of Total Protein from Plant Tissue

Samples were collected and shock frozen in liquid nitrogen. After grinding, the powder was dissolved in 1 μl extraction buffer (150 mM NaCl, 50 mM Tris, 0.1% Tween-20, 10% gylcerol, 1 mM DTT, 1 mM Pefabloc SC (Roche), 1× Complete Proteinase Inhibitior Cocktail [Roche], 1× Halt Phosphatase Inhibitor Cocktail [Pierce]) per mg tissue and mixed briefly. After centrifugation (45 min, 4°C, 20,000 rcf), the supernatant was transferred to a fresh tube and the protein content determined by Bio-Rad Bradford assay. All samples were stored at −20°C until further analysis.

Protein Analysis

20 μg total protein extract were mixed with 5× Laemmli buffer (20 mM Tris, 1% SDS, 0.05% bromphenolblue, 10% glycerol [pH 6.8]) and incubated at 96°C for 5 min. Depending on the theoretical size of the proteins under investigation, the samples were then resolved on 10% or 12% SDS-containing polyacrylamide gels [36]. The electrophoresis was conducted at a constant current of 20 mA. Proteins were transferred onto PVDF membrane (Bio-Rad) with a Panther semidry blotting device (Peqlab). Transfer was conducted at a constant 1 mA/cm^2 membrane surface.

After transfer of the proteins, the membrane was saturated with 5% skim milk powder (Merck) in TBST (10 mM Tris, 150 mM NaCl, 0.2% Tween-20 [pH 7.5]). Incubation with the first antibody (polyclonal rabbit anti-GFP antibody, ChIP Grade, Abcam) was performed overnight at 4°C; after washing three times in TBST, incubation with the secondary antibody (polyclonal donkey anti-rabbit-HRP; Dianova) in TBST was performed for 1 hr at room temperature. Bound

antibody was detected with the ECL Detection Kit (Amersham) and BioMax MS films (Kodak).

TEV Protease Cleavage-Site Prediction

The *Arabidopsis* proteome was searched for the occurrence of the canonical TEV protease recognition site (ENLYFQ|G) with Patmatch at TAIR (http://www.Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl).

Microscopy

Plants were examined for expression YFP with a Leica MZ FLIII microscope fitted with wide- and band-pass YFP filters and a AxioCam HRc (Zeiss) digital camera with AxioVison software (version 3.1; Zeiss). Confocal YFP images (Figure S2) were acquired with the 514 nm excitation line of an argon laser on a Leica TCS SP2 confocal microscope at 10× magnification.

qRT-PCR

Total RNA was isolated from leaves with TRIZOL reagent (Invitrogen). Reverse transcription was performed with 2 μg RNA via the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR was carried out in the Opticon Continuous Fluorescence Detection System with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Expression was determined in relation to tubulin. The following primers were used: Tubulin, 5′-gag cct tac aac gct act ctg tct gtc-3′ and 5′-aca cca gac ata gta gca gaa atc aag-3′; TEV-Protease, 5′-gca cat tcc ctt cat ctg-3′ and 5′-caa caa tga acc cat ctc-3′.

Supplemental Data

Two figures and one table are available at http://www.current-biology.com/cgi/content/full/17/12/1055/DC1/.

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