The Putative O-Linked N-Acetylglucosamine Transferase SPINDLY Inhibits Class I TCP Proteolysis to Promote Sensitivity to Cytokinin[1]

Evyatar Steiner, Sivan Livne, Tammy Kobinson-Katz, Lior Tal, Oded Pri-Tal, Assaf Mosquna, Danuše Tarkowská, Bruno Mueller, Petr Tarkowski, and David Weiss*

Institute of Plant Sciences and Genetics in Agriculture, Hebrew University of Jerusalem, Rehovot 76100, Israel (E.S., S.L., T.K.-K., O.P.-T., A.M., D.W.); Department of Plant Sciences, Weizmann Institute of Science, Rehovot 76100, Israel (L.T.); Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Metabolomics, Institute of Experimental Botany, CZ–78371 Olomouc, Czech Republic (D.T.); Department of Plant and Microbial Biology, University of Zurich, Zurich-Basel Plant Science Center, 8008 Zurich, Switzerland (B.M.); and Centre of the Region Haná for Biotechnological and Agricultural Research, Central Laboratories and Research Support, Faculty of Science, Palacky University, and Department of Genetic Resources for Vegetables, Medicinal, and Special Plants, Crop Research Institute, CZ–78371 Olomouc, Czech Republic (P.T.)

ORCID IDs: 0000-0003-0467-9287 (A.M.); 0000-0003-1478-1904 (D.T.); 0000-0002-3253-8441 (D.W.).

Arabidopsis (*Arabidopsis thaliana*) SPINDLY (SPY) is a putative serine and threonine *O*-linked *N*-acetylglucosamine transferase (OGT). While SPY has been shown to suppress gibberellin signaling and to promote cytokinin (CK) responses, its catalytic OGT activity was never demonstrated and its effect on protein fate is not known. We previously showed that SPY interacts physically and functionally with TCP14 and TCP15 to promote CK responses. Here, we aimed to identify how SPY regulates TCP14/15 activities and how these TCPs promote CK responses. We show that SPY activity is required for TCP14 stability. Mutation in the putative OGT domain of SPY (*spy-3*) stimulated TCP14 proteolysis by the 26S proteasome, which was reversed by mutation in CULLIN1 (CUL1), suggesting a role for SKP, CUL1, F-box E3 ubiquitin ligase in TCP14 proteolysis. TCP14 proteolysis in *spy-3* suppressed all *TCP14* misexpression phenotypes, including the enhanced CK responses. The increased CK activity in TCP14/15-overexpressing flowers resulted from increased sensitivity to the hormone and not from higher CK levels. *TCP15* overexpression enhanced the response of the CK-induced synthetic promoter *pTCS* to CK, suggesting that TCP14/15 affect early steps in CK signaling. We propose that posttranslational modification of TCP14/15 by SPY inhibits their proteolysis and that the accumulated proteins promote the activity of the CK phosphorelay cascade in developing Arabidopsis leaves and flowers.

O-linked GlcNAc (*O*-GlcNAc) modification of Ser and Thr residues by the nucleocytoplasmic *O*-GlcNAc transferases (OGTs) regulates the posttranslational fate and function of target proteins (Hart et al., 2007; Butkinaree et al., 2010). In mammalian cells, *O*-GlcNAcylation affects protein localization, phosphorylation, and stability and plays a role in signal transduction, transcription, and proteasomal degradation (Roos and Hanover, 2000; Wells et al., 2001; Hanover, 2001; Hanover et al., 2003, 2010; Zachara and Hart, 2004; Yang et al., 2008; Butkinaree et al., 2010). Although similar modification occurs in plants, only a few *O*-GlcNAcylated plant proteins have been identified. These include the tobacco (*Nicotiana tabacum*) histone protein and the nuclear pore protein gp40 (Heese-Peck et al., 1995; Heese-Peck and Raikhel, 1998; Schouppe et al., 2011), the wheat (*Triticum aestivum*) RNA-binding protein TaGRP2 (Xiao et al., 2014), and the Arabidopsis (*Arabidopsis thaliana*) GA signaling suppressor DELLAs (Zentella et al., 2016). *O*-GlcNAcylation of TaGRP2 and DELLA affects their interaction with other proteins (Xiao et al., 2014; Zentella et al., 2016), but other effects typically associated with this modification in mammals, including stability, have yet to be discovered in plants.

The Arabidopsis genome encodes two putative OGTs, SPINDLY (SPY) and SECRET AGENT (SEC). The OGT activity of SEC has been demonstrated (Kim et al., 2011), and a recent study by Zentella et al. (2016) showed that SEC interacts with and *O*-GlcNAcylates DELLAs, reduces their activity, and promotes GA

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* Address correspondence to david.weiss@mail.huji.ac.il.

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responses. SPY, similar to SEC, contains multiple tetratricopeptide repeats at the N terminus, which bind substrate proteins, and a putative OGT catalytic domain at the C terminus (Silverstone et al., 2007). Overexpressing SPY's N terminus without the catalytic OGT domain creates a dominant-negative effect (i.e. a *spy*-like phenotype; Izhaki et al., 2001; Tseng et al., 2001). It was proposed that the highly expressed tetratricopeptide repeat domain competes with the endogenous intact, but less abundant, SPY for interaction with substrate proteins. The catalytic OGT domain of SPY comprises CD I and CD II, two conserved regions that form a UDP-GlcNAc-binding pocket that catalyzes the transfer of GlcNAc monosaccharide to the substrate proteins in an *O*-linkage (Roos and Hanover, 2000). Despite the similarity in protein structure, the OGT activity of SPY was never demonstrated; thus, it is still not clear if SPY is a true OGT.

SPY has been implicated in various aspects of plant growth and development, including hormone responses (Olszewski et al., 2010). SPY is a negative regulator of GA signaling (Jacobsen and Olszewski, 1993). Indirect evidence suggested that SPY activates the DELLA proteins, thereby suppressing GA responses (Olszewski et al., 2010). Zentella et al. (2016) showed that SPY interacts with DELLAs, but they did not find evidence for DELLA *O*-GlcNAcylation by SPY. Thus, the mechanism by which SPY suppresses GA responses is still not clear.

We showed previously that SPY promotes cytokinin (CK) responses in developing leaves and flowers (Greenboim-Wainberg et al., 2005; Maymon et al., 2009). Two *spy* alleles showing severe (*spy-4*) and mild (*spy-3*) GA-associated phenotypes (Filardo and Swain, 2003) exhibited similar resistance to CK, suggesting that SPY enhances CK responses and inhibits GA signaling through distinct pathways (Greenboim-Wainberg et al., 2005). The CK signaling pathway starts with binding of CK to His kinase (HK, or AHK in Arabidopsis) receptors, which are then autophosphorylated. The phosphate group is then transferred by His phosphotransfer proteins (Hpt, or AHP in Arabidopsis) to the nucleus, where it phosphorylates and activates a set of transcriptional regulators known as type B response regulators (RRs, or ARR in Arabidopsis). The phosphorylated type B RRs promote the transcription of various CK-regulated genes, including type A *RR*s, which, in turn, suppress CK responses (Müller and Sheen, 2007; Hwang et al., 2012). We found that SPY interacts with and activates the class I TCP transcription factors, TCP14 and TCP15, to promote CK responses (Steiner et al., 2012). Since both *spy* and *tcp14 tcp15* loss-of-function mutants suppressed various CK responses in leaves and flowers, including the expression of type A *ARR* genes, we speculated that SPY and TCPs either promote CK accumulation or increase the activity of components in the CK phosphorelay cascade (Greenboim-Wainberg et al., 2005; Steiner et al., 2012).

TCPs belong to the family of basic helix-loop-helix-type transcription factors. The Arabidopsis genome encodes 24 predicted TCP proteins, 13 of which are grouped as class I and 11 as class II (Martín-Trillo and Cubas, 2010). Class I TCPs have been suggested to promote, and class II to restrict, cell proliferation (Nath et al., 2003; Li et al., 2005, 2012; Efroni et al., 2008). Most class I TCP single mutants have mild or no phenotypes, probably because of genetic redundancy (Martín-Trillo and Cubas, 2010). However, several studies showed a role for these proteins in cell proliferation and organ development. TCP20 binds to the promoters of *CYCLIN B1;1* and promotes cell division (Li et al., 2005). TCP15 suppresses endoreduplication by regulating the expression of cell cycle genes (Li et al., 2012). Kieffer et al. (2011) demonstrated the redundant activity of TCP14 and TCP15 in the regulation of cell proliferation during leaf development. Davière et al. (2014) showed that TCP8, TCP14, TCP15, and TCP22 interact with DELLA proteins and suggested that DELLAs inhibit their promoting effect on cell division and stem elongation. Recently, Lucero et al. (2015) showed that TCP15 affects gynoecium development and suggested its role in a feedback loop, regulating the balance between auxin and CK.

Here, we show that SPY activity is required for TCP14 accumulation. Mutation in the putative OGT catalytic domain of SPY promoted TCP14 proteolysis by the 26S proteasome and suppressed CK signaling.

RESULTS

The OGT Catalytic Domain of SPY Is Required for TCP14 Activity

We previously showed that SPY physically and functionally interacts with TCP14 and TCP15 (Steiner et al., 2012). To understand the mechanism by which SPY promotes TCP14/15 activity, we tested the role of SPY's putative OGT catalytic domain in TCP14 activity. To this end, we overexpressed TCP14 in *spy-3*, which is a weak allele of SPY, caused by a single amino acid substitution (Gly to Ser, at position 593 at the CD I motif) in the putative OGT catalytic domain (Silverstone et al., 2007). A similar amino acid substitution in the corresponding human OGT completely abolished its OGT catalytic activity (Lazarus et al., 2005). We first expressed GFP-TCP14 under the regulation of the *AS1* promoter (Steiner et al., 2012) in wild-type Columbia (Col; *pAS1*:*GFP-TCP14*). *pAS1*:*GFP-TCP14* plants had small dark leaves, short narrow petals, an increased number of trichomes on sepals, and small siliques (Fig. 1, A–C). In addition, seed number in *pAS1*:*GFP-TCP14* pods was strongly reduced (Fig. 1D), resulting from the long carpels protruding from the stigma above the stamens (Fig. 1B), which partially prevented self-pollination. Hand pollination of *pAS1*:*GFP-TCP14* wild-type flowers restored normal silique development (Supplemental Fig. S1). We then introgressed the transgene (*pAS1*:*GFP-TCP14*) into *spy-3* plants. *spy-3* strongly suppressed all TCP14 overexpression phenotypes (Fig. 1), suggesting that SPY's OGT domain is essential for TCP14 activity. *GFP-TCP14* mRNA levels in the transgenic wild type

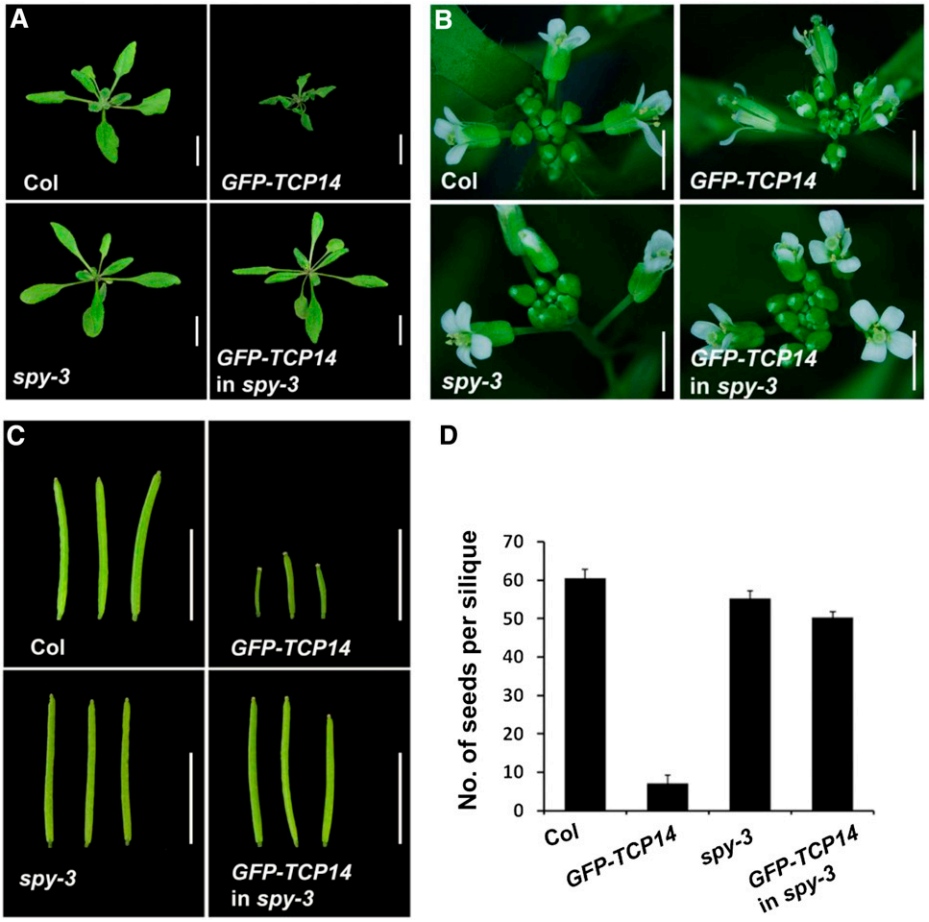

Figure 1. *spy-3* suppresses TCP14 activity. Phenotypic characterization of wild-type Col and *spy-3* as well as transgenic Col (*GFP-TCP14*) and *spy-3* (*GFP-TCP14* in *spy-3*) overexpressing *GFP-TCP14* under the regulation of the *AS1* promoter. A, Three-week-old plants grown under long-day conditions. B, Inflorescences. C, Siliques. Bars = 1 cm. D, Average seed number per silique, determined from 10 pods, ± SE.

and *spy-3* were found to be similar (Fig. 2C), suggesting that *spy-3* affects TCP14 at the posttranscriptional level.

We then examined whether SPY also affects class II TCPs. To this end, transgenic plants expressing *rTCP4-GFP* under the control of the *BLS* promoter (Efroni et al., 2013) were crossed with *spy-3* (for introgression of the transgene), and the TCP4 overexpression phenotype in wild-type Col versus *spy-3* backgrounds was compared. TCP4 overexpression affecting leaf form was observed in both the wild-type and *spy-3* backgrounds (Supplemental Fig. S2), suggesting that SPY has no effect on TCP4 activity.

SPY Affects TCP14 Stability

We next studied how SPY promotes TCP14 activity. *O*-GlcNAc modifications in mammalian cells regulate the posttranslational fate of target proteins, including cellular localization and stability (Roos and Hanover, 2000; Wells et al., 2001; Hanover et al., 2003; Butkinaree et al., 2010). We examined whether the OGT domain of SPY is required for the regulation of TCP14 localization and/or accumulation. To this end, we imaged the homozygous transgenic *pAS1*:*GFP-TCP14* wild-type and *spy-3* plants described above, using confocal microscopy. In both backgrounds, the GFP signal was found in nuclei, but it was much stronger in wild-type than in *spy-3* plants (Fig. 2A). These findings suggest that TCP14 accumulation, but not localization, is affected by SPY. To confirm these results, proteins were extracted from young inflorescences of nontransgenic and transgenic wild-type and *spy-3* plants expressing GFP-TCP14 to determine the level of the recombinant protein using an anti-GFP antibody. The GFP-TCP14 level was much higher in transgenic wild-type than *spy-3* inflorescences (Fig. 2B). Since *GFP-TCP14* mRNA levels were similar in transgenic wild-type and *spy-3* inflorescences (Fig. 2C), it can be concluded that *spy-3* plays a posttranscriptional role in GFP-TCP14 regulation, likely on protein stability.

To rule out the possibility that *spy-3* affects GFP and not TCP14 accumulation/stability, we expressed *GFP-AHP2* under the control of the *35S* promoter in the wild type and *spy-3* (*35S*:*GFP-AHP2* was transformed into wild-type Col and then introgressed into *spy-3*) and analyzed GFP-AHP2 levels. Western-blot analysis using anti-GFP antibody showed similar levels of the detected protein in *spy-3* and the wild type (Supplemental Fig. S3), suggesting that SPY specifically affects TCP14 and not GFP accumulation/stability.

We further investigated whether TCP14 is biodegraded by the 26S proteasome in *spy-3*. Young transgenic *spy-3* flowers were immersed, for 12 h, in a

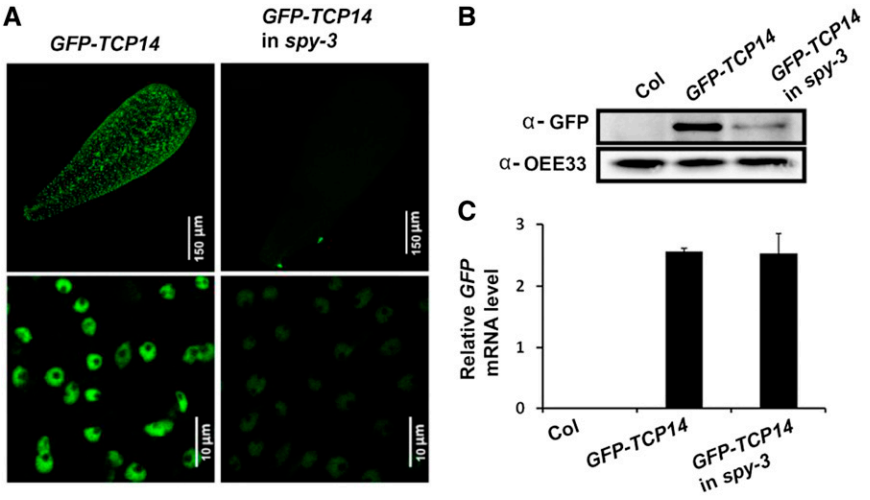

Figure 2. The SPY OGT domain is required for TCP14 protein accumulation. A, Confocal microscopy images of transgenic *pAS1*:*GFP-TCP14* wild-type Col (*GFP-TCP14*) and *spy-3* (*GFP-TCP14* in *spy-3*) petals. Bottom images show petal nuclei. B, Western-blot analysis of proteins extracted from control nontransgenic Col as well as transgenic *pAS1*:*GFP-TCP14* Col and *spy-3* inflorescences. GFP-TCP14 was detected using anti-GFP antibody. OEE33 served as a control to ensure equal loading (Lindahl et al., 1996). C, Quantitative reverse transcription (qRT)-PCR analysis of *GFP* expression in inflorescences of nontransgenic Col as well as transgenic *pAS1*:*GFP-TCP14* Col and *spy-3*. Values are averages of three biological replicates ± SE.

solution containing 50 μM of the 26S proteasome inhibitor MG132 before GFP-TCP14 accumulation was analyzed by confocal microscopy. MG132 treatment promoted the accumulation of GFP-TCP14 in *spy-3* petals (Fig. 3A). Immunoblot analysis of proteins extracted from young inflorescences treated as above confirmed the effect of MG132 (Fig. 3B). These results suggest that SPY stabilizes TCP14 and prevents its proteolysis by the 26S proteasome.

Since TCP14 activity is promoted by CK (Steiner et al., 2012; Lucero et al., 2015), we examined whether CK also affects TCP14 accumulation. Confocal microscopy analysis of CK-treated *pAS1*:*GFP-TCP14* wild-type petals revealed that the hormone has no effect on GFP-TCP14 level (Supplemental Fig. S4).

To determine whether TCP14 proteolysis is mediated by the SKP, CULLIN (CUL), F-box (SCF)-containing E3 ubiquitin ligase complex, we tested the effect of a mutation in CUL1 on TCP14 stability in *spy-3*. We introgressed both *pAS1*:*GFP-TCP14* and *spy-3* into the weak *cul1-6* allele (Moon et al., 2007) background and generated homozygous *spy-3 cul1-6*-expressing *GFP-TCP14* plants. *TCP14* overexpression suppressed silique and petal development in the wild type, an effect that was reversed in *spy-3*, which presented normal siliques and petals (Fig. 4, A and B). *pAS1*:*GFP-TCP14* strongly suppressed silique and petal development in the *spy-3 cul1-6* homozygous mutant background, suggesting that TCP14 is active in the *cul1-6 spy-3* background. Confocal microscopy analysis showed that GFP-TCP14 accumulation was restored in the *cul1-6 spy-3* background (Fig. 4C). These results suggest that TCP14 proteolysis in *spy-3* is mediated by the SCF E3 ubiquitin ligase complex.

The Catalytic OGT Domain of SPY Is Required for TCP14-Dependent Enhancement of CK Responses

We previously showed that TCP14 and TCP15 promote CK responses (Steiner et al., 2012). Here, we investigated whether the catalytic OGT domain of SPY is required for the promotion of CK responses by TCP14. Seedlings of wild-type Col, *pAS1*:*GFP-TCP14*, *spy-3*, and *pAS1*:*GFP-TCP14* in *spy-3* were treated with different concentrations of N^6-benzyladenine (BA) twice per week for 1 month to determine the effect of CK on flower and leaf morphology. Treatment with 10 μM BA reduced petal size in wild-type Col and had an even stronger effect in *TCP14*-overexpressing flowers (Fig. 5A). *spy-3* completely blocked this effect in nontransgenic and transgenic *pAS1*:*GFP-TCP14* flowers.

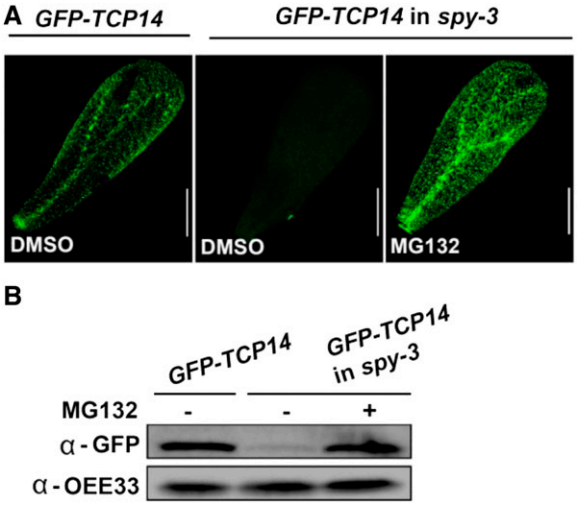

Figure 3. TCP14 is destroyed in *spy-3* by the 26S proteasome. A, Confocal analysis of GFP in transgenic *pAS1*:*GFP-TCP14* wild-type Col (*GFP-TCP14*) and *spy-3* (*GFP-TCP14* in *spy-3*) petals treated with 50 μM MG132 or dimethyl sulfoxide (DMSO). Bars = 200 μm. B, Western-blot analysis of proteins extracted from young inflorescences of transgenic *pAS1*:*GFP-TCP14* wild-type Col and *spy-3* treated with 50 μM MG132 (+) or dimethyl sulfoxide (−). The recombinant GFP-TCP14 protein was detected using an anti-GFP antibody. OEE33 was used as a loading control.

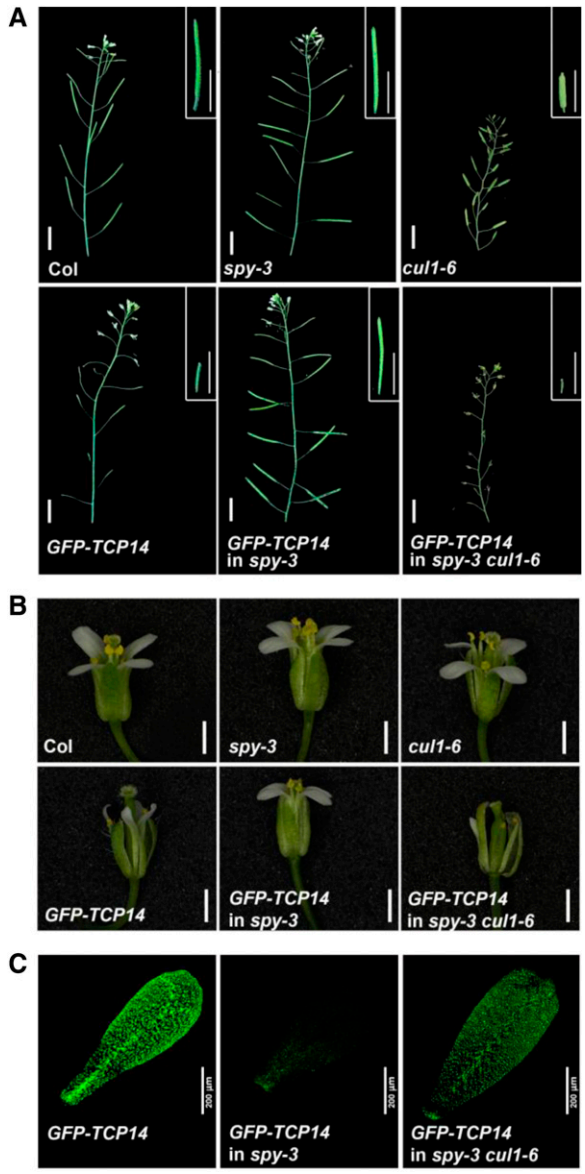

Figure 4. *cul1-6* restores TCP14 activity and stability in a *spy-3* background. A, Mature inflorescences of wild-type Col, *spy-3*, and *cul1-6* and of transgenic *pAS1*:*GFP-TCP14* wild-type Col (*GFP-TCP14*), *spy-3* (*GFP-TCP14* in *spy-3*), and *spy-3 cul1-6* (*GFP-TCP14* in *spy-3 cul1-6*). Insets show a representative silique of each plant. Bars = 1 cm. B, Flowers of the different lines. Bars = 1 mm. C, Confocal analysis of GFP in petals of transgenic *pAS1*:*GFP-TCP14* wild-type Col, *spy-3*, and *spy-3 cul1-6*.

Treatment with a higher CK concentration (50 μM BA) strongly suppressed sepal, petal, stamen, and carpel growth in wild-type Col. The effect was stronger in *TCP14*-overexpressing flowers, except for the carpels, which were hardly affected by the CK treatment. All of these CK effects in the flowers were strongly suppressed by *spy-3* (Fig. 5A). BA treatment (50 μM) also affected leaf morphology, as manifested by smaller and serrated wild-type leaves and an even more exaggerated phenotype in *pAS1*:*GFP-TCP14* (Fig. 5B). Again, *spy-3* suppressed these CK effects. Taken together, these observations suggest that TCP14 activity in leaves and flowers promotes CK responses (except for the carpels) in a SPY-dependent manner and that the SPY catalytic OGT domain is required for this effect.

TCP14 and TCP15 Promote Primary CK Responses

To understand how TCP14 and TCP15 promote CK responses, we first analyzed CK levels in flowers of wild-type Col and *pAS1*:*GFP-TCP14* plants. In Arabidopsis, the major active CKs are N^6-(Δ^2-isopentenyl) adenine and trans-zeatin (Sakakibara, 2006; Frébort et al., 2011). Our analysis showed that the levels of these two active CKs, as well as those of cis-zeatin and dihydrozeatin, were reduced significantly in TCP14-overexpressing inflorescences (Table I). In contrast, the levels of several inactive CKs were increased in the transgenic plants (Supplemental Table S1). These results suggest that TCP14 does not promote CK responses by increasing CK levels but seemingly by promoting hormone signaling. It is likely that the reduced levels of active CK in TCP14-overexpressing flowers result from a negative feedback loop initiated by the increased CK signaling (Kieber and Schaller, 2014).

TCP14 and TCP15 may affect CK activity by promoting early CK signaling events or by inducing the transcription of genes downstream to the CK phosphorelay cascade. To distinguish between these possibilities, we analyzed the effect of TCP15 activity on the synthetic CK-responsive promoter-reporter *TWO-COMPONENT-OUTPUT SENSOR VERSION2* (*TCSv2*). This CK reporter is a variant of TCSn (Zürcher et al., 2013) that harbors the concatemerized type B ARR-binding motifs in alternating head-to-head and tail-to-tail orientations and a minimal *35S* promoter sequence and, thus, reflects the activity of the CK phosphorelay cascade. We fused *TCSv2* to three tandem repeats of the nuclear yellow fluorescent protein (YFP), VENUS (Heisler et al., 2005), to generate *TCSv2*:*3xVENUS-N7*, which we then transformed into Landsberg *erecta* (Ler) plants. Transgenic lines showing moderate YFP signal in the shoot apical meristem were selected for crossing with *TCP15*-overexpressing plants (*pAS1* > >*TCP15*; Steiner et al., 2012) to generate *pAS1* > >*TCP15 TCSv2*:*3xVENUS-N7* plants. One-month-old transgenic *TCSv2*:*3xVENUS-N7* wild-type and *pAS1* > >*TCP15* plants were sprayed with different CK (BA) concentrations, and 24 h later, *TCS* activity (YFP signal) was analyzed in young inflorescences by confocal microscopy. *TCS* activity was promoted in *pAS1* > >*TCP15* inflorescences following treatment with 1 μM BA and was further augmented in a dose-dependent manner (Fig. 6). In the wild-type background, *TCS* activity was only visually detectable after treatment with 10 μM BA, and although the signal was enhanced with higher BA concentration, it was much lower than that detected in *TCP15*-overexpressing inflorescences. These results suggest that *TCP15* overexpression

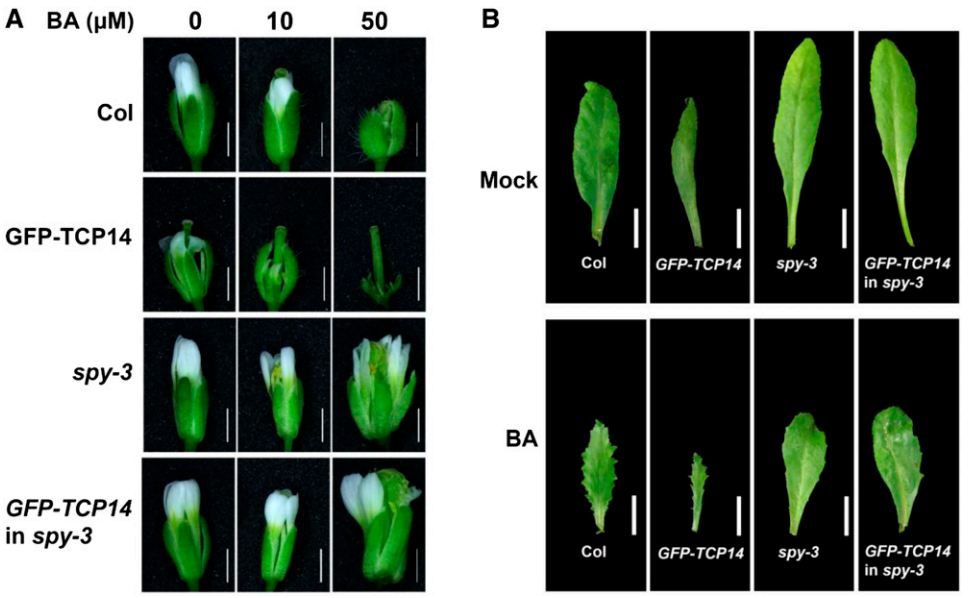

Figure 5. *spy-3* suppresses TCP14-stimulated CK responses. A, Flowers of wild-type Col, *pAS1*:*GFP-TCP14* (*GFP-TCP14*), *spy-3*, and *pAS1*:*GFP-TCP14* in *spy-3* (*GFP-TCP14* in *spy-3*) following treatment with different concentrations of BA (0, 10, and 50 μM). Bars = 1 mm. B, Rosette leaves (leaf 5) of wild-type Col, *pAS1*:*GFP-TCP14*, *spy-3*, and *pAS1*:*GFP-TCP14* in *spy-3* following treatment of young seedlings with two true leaves with 50 μM BA or water (Mock) twice per week for 1 month. Bars = 1 cm.

increases the sensitivity to CK and that TCP14 and TCP15 promote the early stages of the CK signaling pathway.

TCSv2 harbors the concatemerized type B ARR-binding motifs (Müller and Sheen, 2008; Zürcher et al., 2013), implying that the promoting effect of TCP15 on CK-induced *pTCS* activity is mediated by type B ARRs. Since TCPs are transcription factors, at least three scenarios for the interaction between TCP14/15 and type B ARRs are possible: (1) TCPs promote type B *ARR* gene transcription to promote CK responses; (2) TCP14 and TCP15 interact with type B ARRs and act as transcription coactivators to promote CK responses; and (3) TCPs indirectly promote type B ARR activity. The type B ARR family contains 11 proteins, among which ARR1, ARR10, and ARR12 play central yet redundant roles in CK signal transduction (Mason et al., 2005; Ishida et al., 2008). Expression analysis of *ARR1*, *ARR10*, *ARR12*, and *ARR14* in *pAS1* > >*TCP14* (Steiner et al., 2012) and *pAS1* > > *TCP15* flowers suggests that both TCPs have no effect on type B ARR transcription (Supplemental Fig. S5). We also performed a yeast two-hybrid assay to test for a possible interaction between TCP14 and *ARR1*, *ARR10*, and *ARR12*. SPY and AHP2 served as positive controls for the interaction with TCP14 and with type B ARRs, respectively (Dortay et al., 2006; Steiner et al., 2012). Indeed, while TCP14 interacted with SPY and all tested type B ARRs interacted with AHP2, no interaction between TCP14 and any tested type B ARRs was observed (Supplemental Fig. S6). These results imply that TCP14 and TCP15 indirectly promote type B ARR activity. To examine if TCP14 and TCP15 affect the transcription of components upstream of type B ARRs in the CK phosphorelay cascade, we analyzed the expression of the CK receptor kinase genes *AHK2*, *AHK3*, and *AHK4* in wild-type (Col and L*er*), *TCP14*-overexpressing (Col), and *TCP15*-overexpressing (L*er*) inflorescences. qRT-PCR analyses indicated that both TCPs have no effect on the expression of these *AHK* genes (Supplemental Fig. S7).

DISCUSSION

Previously, we showed that SPY interacts physically and functionally with TCP14 and TCP15 and that the null *spy-4* allele suppresses the activity of both TCPs (Steiner et al., 2012). Due to the significant similarity between SPY and animal OGTs (Olszewski et al., 2010), we speculated that SPY *O*-GlcNAcylates TCP14 and TCP15 to promote their activity (Steiner et al., 2012). This hypothesis was tested here using the weak *spy-3* allele, which has a single amino acid substitution (Gly-593 to Ser) in the putative OGT catalytic domain (Silverstone et al., 2007). This specific amino acid (Gly-593) is essential for the catalytic activity of OGTs, as mutation at the corresponding residue of the human OGT completely abolished its OGT activity (Lazarus et al., 2005). In *spy-3*, TCP14 activity was strongly suppressed, suggesting that the putative OGT catalytic domain of SPY is essential for TCP14 activity. Since the OGT domain is not required for the interaction of SPY with TCP14 or TCP15 (Steiner et al., 2012), it is possible that it modifies them, perhaps with GlcNAc.

O-GlcNAcylation in mammalian cells affects protein stability. For example, *O*-GlcNAcylation of the tumor suppressor p53 protects the protein from proteolysis by the proteasome (Yang et al., 2006). We found that a mutation in SPY's OGT catalytic domain dramatically reduces the level of the chimeric GFP-TCP14 protein. Since the accumulation of GFP-TCP14 in *spy-3* was recovered by mutation in CUL1 and by the 26S proteasome

Table I. *Effect of TCP14 overexpression on the accumulation of free-base CKs*

Mass spectrometric measurements were made of free-base CKs in Col and *pAS1:GFP-TCP14* (*GFP-TCP14*) inflorescences. iP, N^6-(Δ^2-isopentenyl)adenine; tZ, trans-zeatin; cZ, cis-zeatin; DHZ, dihydrozeatin. Values are means of three biological replicates ± SE. ND, Not detected

Genotype	CK Content			
	iP	tZ	cZ	DHZ
	pg mg^{-1} fresh wt			
Col	1.12 ± 0.092	0.2 ± 0.003	0.27 ± 0.011	0.01 ± 0.001
GFP-TCP14	0.27 ± 0.025	0.16 ± 0.013	0.03 ± 0.005	ND

inhibitor MG132, we suggest that in *spy*, TCP14 is ubiquitinated by an SCF E3 ubiquitin ligase and consequently destroyed in the proteasome. The results of Peng et al. (2015), showing that TCP14 and TCP15 interact with the ubiquitin receptors DA1, DAR1, and DAR2 for proteolysis, support our suggestion.

We previously showed that SPY promotes CK responses in leaves and flowers (Greenboim-Wainberg et al., 2005) and suggested that this effect is mediated by TCP14 and TCP15 (Steiner et al., 2012). Here, we show that the effect of TCP14 on CK responses is dependent on SPY's catalytic OGT domain, suggesting that the stabilization of TCP14 by SPY activity is required for the increased CK responses. We also found that the accumulated TCPs act by promoting sensitivity to CK and not by increasing CK content. The level of the central active CK, N^6-(Δ^2-isopentenyl)adenine, was reduced in TCP14-overexpressing flowers. The reduced levels of active CKs might be the result of a negative feedback regulation, initiated by increased CK signaling (Kieber and Schaller, 2014). High CK activity induces the expression of *CYTOKININ OXIDASE/DEHYDROGENASE* genes for CK degradation (Brugière et al., 2003; Werner et al., 2003, 2006) and rapidly converts free active CKs to inactive derivatives (Singh et al., 1988; Moffatt et al., 1991; Yonekura-Sakakibara et al., 2004). Indeed, we found higher levels of inactive CKs in TCP14-overexpressing flowers. Altogether, these results suggest that TCP14 overexpression increases CK signaling and not accumulation. This conclusion is supported by our previous observation that leaves and flowers of the double mutant *tcp14 tcp15* are less sensitive to exogenous CK (Steiner et al., 2012). We cannot exclude, however, the possibility that TCP14 and TCP15 promote transient increases in CK level at a very early stage of flower development.

TCP14 and TCP15 may promote sensitivity to CK by affecting components of the early phases of CK signaling or by promoting the expression of downstream genes. Since *TCP15* overexpression increases the sensitivity of the synthetic CK-induced promoter *pTCS* to CK, we propose that TCP14/15 affect element(s) in the early stages of CK signaling. *pTCS* harbors the concatemerized type B ARR-binding motifs (Müller and Sheen, 2008; Zürcher et al., 2013) but not a TCP-binding element, suggesting that TCP15 promotes *TCS* activity via type B ARRs. As TCP14 and TCP15 did not promote type B ARR gene expression and did not interact with type B ARR proteins in yeast (*Saccharomyces cerevisiae*), the TCP14/15-driven promotion of type B ARR activity is seemingly indirect.

While it is possible that TCP14 and TCP15 promote the activity of elements upstream of type B ARRs in the CK phosphorelay cascade, we did not find an effect on the expression of the CK receptor genes *AHK2*, *AHK3*, and *AHK4*. It is also possible that TCP14/15 promote type B ARR activity indirectly via the interaction with other signaling pathways. For example, a previous study showed that TCP15 suppresses auxin activity (Lucero et al., 2015). Since auxin interacts negatively with CK (Müller and Sheen, 2008), the inhibition of auxin activity may promote the activity of the CK phosphorelay cascade to promote type B ARR activity. The possible role of type B ARRs in mediating TCP14/15 activity can explain the requirement of CK for TCP14 activity (Steiner et al., 2012); without CK, type B ARRs are not active (Sakai et al., 2001; Müller and Sheen, 2007).

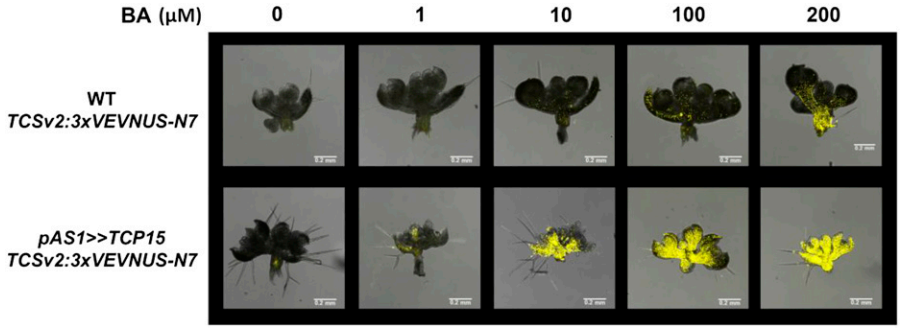

Figure 6. Overexpression of *TCP15* increases sensitivity to CK. Confocal microscopy images show *TCSv2:3xVENUS-N7* in transgenic wild-type (WT) L*er* and *TCP15*-overexpressing (*pAS1 > >TCP15*) plants. One-month-old plants were sprayed with different concentrations of BA, and after 24 h, the YFP signal was detected in young inflorescences.

The results of this and other studies suggest that SPY regulates GA responses and CK activity by different mechanisms. While *spy-3* has only a mild effect on GA responses (Jacobsen and Olszewski, 1993), it has a strong effect, similar to that of the null *spy-4* allele, on CK responses (Greenboim-Wainberg et al., 2005). Thus, while SPY may regulate TCP14/15 and CK responses via glycosylation, the mechanism by which it affects DELLA activity and GA responses is likely to be more complicated and to involve activities other than glycosyltransferase.

A critical question left open is whether SPY indeed *O*-GlcNAcylates TCP14/15 for stabilization. Since all efforts to demonstrate *O*-GlcNAcylation by SPY have failed so far (Zentella et al., 2016), it is possible that the catalytic OGT domain of SPY acquired novel glycosyltransferase activity that affects protein fate similar to *O*-GlcNAcylation. However, we cannot exclude the possibility that steric interference by the catalytic domain of SPY protects TCP14/15 from proteolysis

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) *spy-3* mutant plants used in this study, as well as the transgenic *pAS1:GFP-TCP14*, *35S:GFP-AHP2*, and *pBLS:rTCP4-GFP* plants, were all in the Col background. Transgenic *TCSv2:3xVENUS-N7* and *pAS1 > >TCP15 TCSv2:3xVENUS-N7* were in the L*er* background. Arabidopsis seeds were sterilized, cold treated, and germinated on sterile Murashige and Skoog medium or in pots. The plants were grown in a growth room under controlled temperature (22°C) and long-day (16-h-light/8-h-dark) or short-day (8-h-light/16-h-dark) conditions.

Molecular Cloning/Constructs

The *TCP14*-encoding region was fused to the 3′ end of the enhanced *GFP*-encoding sequence at a *Kpn*I site. The *GFP-TCP14* fusion product was inserted into a pBJ36 plasmid downstream of the *AS1* promoter (Sarojam et al., 2010), between *Xho*I and *Xba*I sites, to create *pAS1:GFP-TCP14*. The construct was subcloned into the pMLBART binary vector, in a *Not*I site, and was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The construct was transformed into Col plants by floral dipping (Clough and Bent, 1998), and BASTA-resistant transformants were selected. The DNA sequence of *TCSv2* was synthesized with flanking *Nsi*I and *Bam*HI restriction sites. The synthetic promoter was then cloned adjacent to *3xVENUS-N7* in the pBJ36 vector (Heisler et al., 2005). The construct was subcloned into the pGREEN binary vector and introduced into the Arabidopsis L*er* background by floral dipping. Kanamycin-resistant transformants were selected.

Immunoblot Analysis

Total protein extracts were obtained by grinding 100 mg of inflorescence tissue in 200 μL of protein extraction buffer (0.2 M Tris-HCl, pH 6.8, 3 M urea, 1% [w/v] glycerol, 8% [w/v] SDS, 0.5 mM dithiothreitol, protease inhibitor cocktail [Sigma-Aldrich], and 5% [w/v] β-mercaptoethanol). Samples were vortexed, boiled for 5 min, and centrifuged at 13,000*g* for 15 min, and the supernatant was subjected to SDS-PAGE. For detection of the GFP-fused proteins, the samples were separated on a 12% polyacrylamide gel in Tris-Gly buffer and electroblotted onto a polyvinylidene difluoride membrane. Blots were reacted with a commercially available anti-GFP antibody (Covance; catalog no. MMS-118R), diluted 1:5,000, and an anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch), diluted 1:5,000. All blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

RNA Purification and qRT-PCR Analyses

Total RNA was extracted from young inflorescences, as described by Shleizer-Burko et al. (2011). qRT-PCR analysis (Table S2) was performed using the Absolute Blue qPCR SYBR Green ROX Mix (AB-4162/B) kit (Thermo Fisher Scientific). Reactions were performed in a Rotor-Gene 6000 cycler (Corbett Research). A standard curve was obtained for each gene, using dilutions of a complementary DNA sample. Each gene was quantified using Corbett Research Rotor-Gene software. At least three independent technical repeats were performed for each complementary DNA sample. The relative expression of each sample was calculated by dividing the expression level of the analyzed gene by that of *TUBULIN BETA CHAIN3*.

CK Analysis

CKs were isolated and purified as outlined by Novák et al. (2003), with some modifications. Frozen Arabidopsis inflorescences (30 mg) were homogenized using a vibration mill (MM 301) at a frequency of 30 Hz for 3 min (3-mm zirconium oxide beads; Retsch), with 1 mL of ice-cold Bieleski solution (methanol: chloroform:formic acid:water, 12:5:1:2) as the extraction solution. The CKs were then extracted overnight at 4°C using a benchtop laboratory rotator (Stuart SB3; Bibby Scientific), after adding heavy-labeled internal standards (OlChemIm). The samples were further fractionated by two steps of solid-phase extraction. Briefly, passing the extracts, in sequence, through a cation (SCX cartridge) and an anion (DEAE-Sephadex combined with a C18 cartridge) exchanger yielded fraction 1, containing the CK bases, ribosides, and glucosides, and fraction 2, containing the riboside-5′-phosphates. Both fractions were further purified by immunoaffinity extraction based on generic monoclonal anti-CK antibodies, but fraction 2 was first treated with alkaline phosphatase. In fraction 1, the *O*-glucosides did not bind to the affinity sorbent. The effluent was thus treated with β-glucosidase to yield the *O*-glucoside fraction (later analyzed as an aglycone). Samples were then redissolved in the mobile phase and analyzed by ultra-HPLC-tandem mass spectrometry (Acquity UPLCTM System, Xevo TQ; Waters MS Technologies) according to Novák et al. (2008). CKs were ionized by electrospray in positive mode and detected using multiple reaction monitoring. Masslynx 4.1 software (Waters) was used to analyze the data.

BA Treatment

Arabidopsis seedlings with two true leaves were sprayed with different concentrations of BA (Sigma-Aldrich) twice per week for approximately 4 weeks. For the *TCSv2* experiments, 1-month-old plants were sprayed once with different concentrations of BA.

MG132 Treatment

Young inflorescences were immersed in solution containing 50 μM MG132 (Sigma-Aldrich) and 0.01% Tween 20, for 12 h in the dark, at room temperature. During the first hour, the samples were vacuumed, and the vacuum was released every 15 min.

The Cytokinin Reporter TCSv2

TCSv2 is a variant of TCSn, with alternating head-to-head and tail-to-tail orientations of type B ARR-binding sites compared with the tandem tail-to-tail and head-to-head orientation of sites in TCSn. TCSv2 mediates CK-dependent activation that is comparable to TCSn. Its sequence is 5′-CAAAGATTTTGCAAAATCTTTTAAAGGATTTTGAAAGATCTTTGCAAAGATCTTTATAAATCTTTTCAAAGATTTTTCAAGATCCGATTAAAGATTTTGCAAAATCTTTAGAGAGATCTTTCAAAATCCAACGCTAGTCAAAGATTTTGCAAAATCTTTTAAAGGATTTTGAAAGATCTTTGCAAAGATCTTTATAAATCTTTTCAAAGATTTTTCAAGATCCGATTAAAGATTTTGCAAAATCTTTAGAGAGATCTTTCAAAATCCAAC-3′.

Microscopy and Confocal Imaging

All imaging was done using a confocal laser scanning microscope (Leica TCS SP8; http://www.leica-microsystems.com/) with an HCX PL APO CS 20×/0.70 dry objective (for GFP in petals) or an HC PL FLUOTAR 10×/0.30 dry objective (for VENUS-YFP in flowers). GFP was excited with the 488-nm laser line, and the 505- to 525-nm filter was used for emission. VENUS-YFP was

excited using the 514-nm laser line in conjunction with a 520- to 560-nm band-pass filter.

Yeast Two-Hybrid Interaction Assay

The coding regions of *ARR1*, *ARR10*, *ARR12*, and *SPY* were cloned into pACT2 plasmid. *TCP14* and *AHP2* were cloned into the pBD-GAL4 CAM plasmid. pACT2-ARR1, pACT2-ARR10, and pACT2-ARR12 were transformed individually into *Saccharomyces cerevisiae* strain Y190 containing pBD-GAL4-TCP14 or pBD-GAL4-AHP2. Yeast transformants were selected for the presence of plasmids on synthetic dextrose agar plates lacking Leu and Trp. Fusion of the GAL4 activating domain and ARR and translational fusion of the TCP14-GAL4 binding domain were confirmed using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid staining to monitor β-gal reporter gene expression levels. Individual clones were spotted onto synthetic dextrose agar plates lacking Leu and Trp. After a 2-d incubation at 30°C, plates were stained using the chloroform overlay 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid method. Empty pACT2 and pBD-GAL4 were used as negative controls, and pACT2- SPY and pBD-GAL4-AHP2 were used as positive controls.

Accession Numbers

The sequences of genes examined in this study can be found in The Arabidopsis Information Resource data library under the following accession numbers: SPY (AT3G11540), TCP14 (AT3G47620), TCP15 (AT1G69690), CUL1 (AT4G02570), TUB3 (AT5G62700), ARR1 (AT3G16857), ARR10 (AT4G31920), ARR12 (AT2G25180), ARR14 (AT2G01760), AHP2 (AT1G13330), AHK2 (AT5G35750), AHK3 (AT1G27320), and AHK4 (AT2G01830).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Hand pollination of *pAS1*:*GFP-TCP14* flowers restored normal silique development.

Supplemental Figure S2. *spy-3* does not suppress the TCP4 overexpression phenotype.

Supplemental Figure S3. *spy-3* does not affect GFP-AHP2 accumulation.

Supplemental Figure S4. CK does not affect GFP-TCP14 stability.

Supplemental Figure S5. Overexpression of *TCP14* or *TCP15* has no effect on type B ARR expression.

Supplemental Figure S6. Type B ARRs do not interact with TCP14 in yeast.

Supplemental Figure S7. Overexpression of *TCP14* or *TCP15* has no effect on *AHK* gene expression.

Supplemental Table S1. Effect of TCP14 overexpression on inactive CK levels.

Supplemental Table S2. List of primers.

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