

# The *Arabidopsis* O-Linked N-Acetylglucosamine Transferase SPINDLY Interacts with Class I TCPs to Facilitate Cytokinin Responses in Leaves and Flowers

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**O-linked N-acetylglucosamine (O-GlcNAc) modifications regulate the posttranslational fate of target proteins. The *Arabidopsis thaliana* O-GlcNAc transferase (OGT) SPINDLY (SPY) suppresses gibberellin signaling and promotes cytokinin (CK) responses by unknown mechanisms. Here, we present evidence that two closely related class I TCP transcription factors, TCP14 and TCP15, act with SPY to promote CK responses. TCP14 and TCP15 interacted with SPY in yeast two-hybrid and in vitro pull-down assays and were O-GlcNAc modified in *Escherichia coli* by the *Arabidopsis* OGT, SECRET AGENT. Overexpression of TCP14 severely affected plant development in a SPY-dependent manner and stimulated typical CK morphological responses, as well as the expression of the CK-regulated gene *RESPONSE REGULATOR5*. TCP14 also promoted the transcriptional activity of the CK-induced mitotic factor *CYCLIN B1;2*. Whereas TCP14-overexpressing plants were hypersensitive to CK, *spy* and *tcp14 tcp15* double mutant leaves and flowers were hyposensitive to the hormone. Reducing CK levels by overexpressing *CK OXIDASE/DEHYDROGENASE3* suppressed the TCP14 overexpression phenotypes, and this suppression was reversed when the plants were treated with exogenous CK. Taken together, we suggest that responses of leaves and flowers to CK are mediated by SPY-dependent TCP14 and TCP15 activities.**

## INTRODUCTION

Addition of O-linked N-acetylglucosamine (O-GlcNAc) to Ser and Thr residues by O-GlcNAc transferases (OGTs) regulates the posttranslational fate and function of target proteins (Hart et al., 2007; Butkinaree et al., 2010). O-GlcNAc modifications of animal cytosolic and nuclear proteins affect their localization, phosphorylation, interaction with other proteins, and/or stability (Roos and Hanover, 2000; Wells et al., 2001; Hanover et al., 2003; Butkinaree et al., 2010). In animals, this modification plays a key role in various cellular processes, such as signal transduction, transcription, and proteasomal degradation (Hanover, 2001; Wells et al., 2003; Zachara and Hart, 2004; Yang et al., 2008; Butkinaree et al., 2010; Hanover et al., 2010). Although a similar modification occurs in plant cells, only a few O-GlcNAc-modified plant proteins have been identified.


The *Arabidopsis thaliana* genome encodes two OGTs, SPINDLY (SPY) and SECRET AGENT (SEC). Whereas *sec* and *spy* embryos


develop normally, double mutant embryos are not viable, suggesting that SEC and SPY have redundant functions during embryogenesis (Hartweck et al., 2002). Although *sec* plants have subtle growth defects (Hartweck and Olszewski, 2006), the pathways in which SEC functions have not been determined. By contrast, SPY functions in the gibberellin (GA), cytokinin (CK), red light response, and circadian pathways (Jacobsen and Olszewski, 1993; Tseng et al., 2004; Greenboim-Wainberg et al., 2005; Maymon et al., 2009). SPY is a negative regulator of GA signaling, and it has been hypothesized that SPY accomplishes this regulation by O-GlcNAc-modifying and activating DELLA proteins, which also negatively regulate GA responses (Shimada et al., 2006; Silverstone et al., 2007). In a previous study, we found that *spy* mutants are partially resistant to exogenously applied CK and proposed that SPY is a positive element in CK signal transduction (Greenboim-Wainberg et al., 2005; Maymon et al., 2009). Because GA also suppressed CK responses, we suggested that crosstalk between these hormones is partially mediated by SPY (Greenboim-Wainberg et al., 2005; Weiss and Ori, 2007). SPY promotes CK responses via a DELLA-independent pathway and thus appears to inhibit GA responses and enhance CK signaling through distinct mechanisms (Maymon et al., 2009).

The CK-signaling cascade in *Arabidopsis* starts with the binding of CK to the His kinase receptors and their autophosphorylation (Müller and Sheen, 2007). The phosphate group is then transferred by His phosphotransfer proteins (AHPs in *Arabidopsis*) to the

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nucleus to phosphorylate a set of transcriptional regulators known as type-B response regulators (RRs; or ARR in *Arabidopsis*). The phosphorylated type-B RRs induce the transcription of various CK-regulated genes, including type-A RRs (D'Agostino et al., 2000; Rashotte et al., 2006). Because GA and *spy* mutations affected numerous CK responses occurring at different stages of plant development, including induction of the CK primary response gene, type-A *Arabidopsis* RRs (*ARR5*; Greenboim-Wainberg et al., 2005; Gan et al., 2007), it was suggested that SPY promotes and GA suppresses elements of the early CK-induced phosphorelay cascade.

To understand the mechanism by which SPY affects plant development, several groups have performed protein interaction assays. These revealed an interaction between SPY and GIGANTEA (GI), which may affect the activity of the circadian clock (Tseng et al., 2004). The *Hordeum vulgare* SPY homolog Hv-SPY was shown to interact with two transcriptional regulators from the MYB- and NAC-like families, which may affect GA responses in this plant (Robertson, 2004). Here, we show that SPY interacts with two class I TCP proteins: TCP14 and its closest homolog TCP15.

TCPs, named after the transcription factors TEOSINTE BRANCHED1 from *Zea mays*, CYCLOIDEA from *Antirrhinum majus*, and PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR from *Oryza sativa* (Navaud et al., 2007), belong to the family of basic helix-loop-helix-type transcription factors. The TCP domain is a plant-specific DNA binding motif that binds specifically to *cis*-elements named site II elements. The *Arabidopsis* genome encodes 24 predicted TCP proteins, of which, based on the structure of the DNA binding domain, 13 are grouped as class I and 11 as class II (Cubas et al., 1999; Kosugi and Ohashi, 2002; Aguilar-Martínez et al., 2007; Navaud et al., 2007). Because the two classes appear to bind similar promoter elements, it was suggested that development is regulated by a balance in the activity of the two groups. Class I TCPs were suggested to promote and class II to restrict cell proliferation (Li et al., 2005; Li et al., 2011).

Class II TCPs affect floral organ asymmetry, axillary meristem development, branching, and leaf shape via the regulation of cell proliferation and/or growth (Martín-Trillo and Cubas, 2010). Some of these effects may be mediated by hormonal activities. TCP1 affects brassinosteroid biosynthesis by regulating the transcription of DWARF4, a key enzyme in brassinosteroid biosynthesis (Guo et al., 2010). TCP3 suppresses auxin responses probably by the transcriptional stimulation of the signaling repressor *INDOLE-3-ACETIC ACID3/SHORT HYPOCOTYL2* (Koyama et al., 2010). TCP4 regulates the transcription of *LIPOXYGENASE2*, a key enzyme in jasmonate biosynthesis (Schommer et al., 2008), and the *Solanum lycopersicum* homolog of TCP4, *LANCEOLATE*, promotes GA biosynthesis (Yanai et al., 2011). TCP13 has been identified in yeast two-hybrid assays as an interactor with AHPs (Suzuki et al., 2001), which raises the possibility that TCP13 is regulated by the CK signal.

Much less is known about the role of class I TCPs in plant development. Most class I single mutants have mild or no phenotypes, probably as a result of genetic redundancy. However, overexpression of *Arabidopsis* TCP20 as a fusion with a transcriptional repressor domain caused phenotypes suggestive of roles in cell division, expansion, and differentiation (Hervé et al., 2009; Martín-Trillo and Cubas, 2010). TCP20 binds a specific motif in the

site II elements (GCCO) found in the promoter of ribosomal protein genes (Tatematsu et al., 2005). TCP21 (CHE) is involved in the regulation of the circadian clock activity (Prunedo-Paz et al., 2009). This protein interacts with a core element of the clock oscillator, PSEUDO RESPONSE REGULATOR1 (PRR1)/TOC1, and together they suppress the transcription of *CIRCADIAN AND CLOCK ASSOCIATED1*. Similarly, TCP15 interacts with the core circadian clock component PRR5 (Giraud et al., 2010), and the diurnal oscillations in *PRR5* transcript abundance were slightly affected in *tcp15* plants. In addition, Li et al. (2011) have shown that TCP15 directly regulates cell cycle genes and inhibits endoreduplication. The *Antirrhinum* TCP14 homolog TIC interacts with a NAC domain transcription factor, CUPULIFORMIS, suggesting a role in the establishment of boundaries between lateral organs (Weir et al., 2004). Tatematsu et al. (2008) have shown that the *Arabidopsis* TCP14 has a role in GA regulation of embryo growth during seed germination and that the *tcp14* mutant seeds are hypersensitive to exogenously applied abscisic acid and paclobutrazol. Recently, Kieffer et al. (2011) characterized the double mutant *tcp14 tcp15* and demonstrated its redundant activities in the regulation of cell proliferation in bolting stem internodes, developing leaf blades, and floral tissues. Here, we show that TCP14 and TCP15 physically and genetically interact with SPY and provide evidence that they act together to promote CK responses in leaves and flowers. These CK responses affect leaf shape and trichome development.

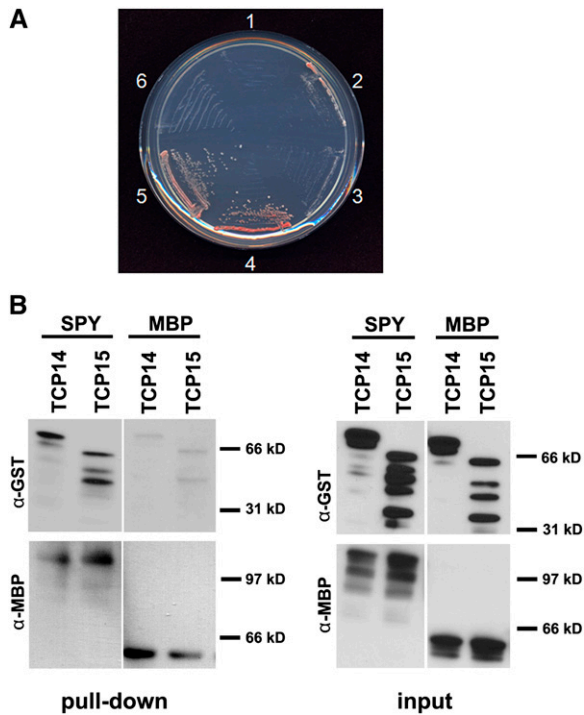
## RESULTS

### SPY Interacts Physically with TCP14 and TCP15

To understand the mechanism by which SPY affects plant development in general and hormone signaling in particular, we performed a yeast two-hybrid screen for SPY-interacting proteins (Tseng et al., 2004). One bait clone expressing a portion of TCP15 was recovered from this screen. Additional yeast two-hybrid assays demonstrated that full-length TCP15 and its closest homolog, TCP14, interacted in this yeast system with the SPY-TPR domain (Figure 1A). Similar to previous reports that TCPs autoactivate in a yeast-based assay (Kosugi and Ohashi, 2002; Giraud et al., 2010), TCP14 and TCP15 prey proteins weakly autoactivated reporter genes, but much stronger activation occurred in the presence of the SPY bait. To provide additional evidence for the physical interaction between SPY and TCP14 and TCP15, we performed *in vitro* pull-down assays using bacterially expressed maltose binding protein (MBP)-tagged full-length SPY and glutathione S-transferase (GST)-tagged TCP14 and TCP15. Whereas very low levels of nonspecific interaction between GST-TCP14/TCP15 and MBP were detected in the pull-down assay, the interaction between full-length GST-TCP14/TCP15 and MBP-SPY was much stronger (Figure 1B). MBP-SPY interacted also with large truncated products of GST-TCP14/15. GST alone did not interact with SPY (see Supplemental Figure 1 online).

### TCP14 Activity Requires SPY

To assess the biological relevance of the physical interaction between SPY and TCP14, we examined whether the activity of TCP14 requires functional SPY. To this end, we first assayed



**Figure 1.** SPY Interacts Physically with TCP14.

**(A)** The results from a yeast two-hybrid assay. Yeast cells containing the following vectors: 1, empty bait and empty prey; 2, empty bait and TCP14 prey; 3, empty bait and TCP15; 4, SPY-TPR bait and TCP14 prey; 5, SPY-TPR bait and TCP15 prey; and 6, SPY-TPR bait and empty prey, were plated on medium containing 10 mM 3-amino-1,2,4-triazole and lacking His. Although cells expressing the TCP14 or TCP15 prey protein alone (2 and 3) exhibited a small amount of growth, cells expressing the TCP bait and SPY-TPR prey proteins (4 and 5) grew much better.

**(B)** In vitro pull-down assay using *E. coli*-expressed MBP, MBP-SPY, GST-TCP14, and GST-TCP15. MBP or MBP-SPY (full-length SPY) were used as bait and incubated with equal amount of GST-TCP14 or GST-TCP15. Amylose resin was used to pull down the MBP or MBP-SPY and its interacting proteins. Left panels (pull-down): An anti-GST antibody was used to detect GST-TCP14 or GST-TCP15. The blot was stripped and reprobed with anti-MBP antibody to detect MBP and MBP-SPY. Right panels (input): Amounts of proteins added to the assays. Full-length GST-TCP14 = 80.1 kD, GST-TCP15 = 62.5 kD, MBP-SPY = 145.7 kD, GST = 26.7 kD, and MBP = 50.8 kD.

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*TCP14* in vivo function by overexpression. Expressing *TCP14* under the regulation of the 35S promoter severely altered plant development and occasionally caused lethality. We therefore expressed *TCP14* under the control of promoters that are active in lateral-organ primordia but not in the shoot apical meristem proper. We evaluated the *FILAMENTOUS FLOWER (FIL)*, *CUP-SHAPED COTYLEDON2 (CUC2)*, and *ASYMMETRIC LEAVES1 (AS1)* promoters using a transactivation system (Moore et al., 1998). The phenotype found in plants expressing *TCP14* under the regulation of the *AS1* promoter (*AS1<sub>pro</sub>>>TCP14*) was stronger than that found in *CUC2<sub>pro</sub>>>TCP14* or *FIL<sub>pro</sub>>>TCP14* plants (Figure 2A; see Supplemental Figure 2 online). Thus, *AS1<sub>pro</sub>>>TCP14* plants were selected for this study. The *AS1<sub>pro</sub>* driver used for transactivation (*AS1<sub>pro</sub>:LhG4*) is active primarily in leaf primordia, young leaves, and

young flowers (Eshed et al., 2001); this activity was confirmed by monitoring the expression of an operator (*op*):*green fluorescent protein (GFP)* transgene that was included in all crosses (*AS1<sub>pro</sub>>>ER-GFP*; see Supplemental Figures 3B and 3C online).

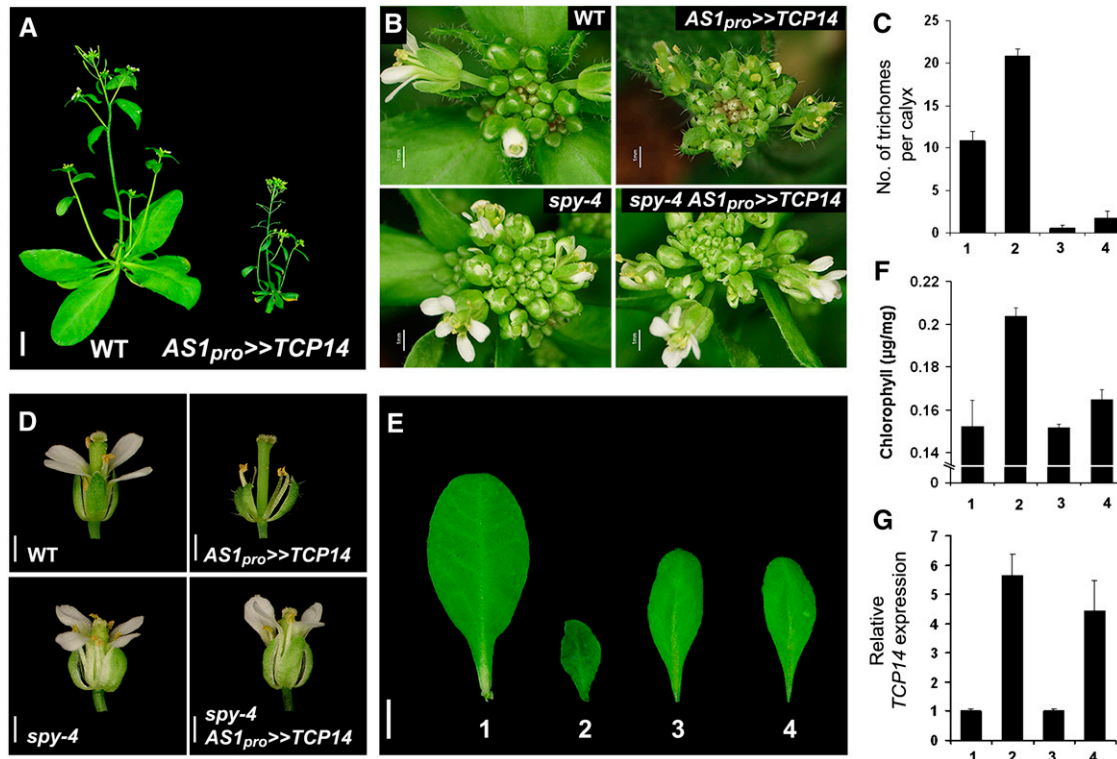
Overexpression of *TCP14* (*AS1<sub>pro</sub>>>TCP14*) resulted in inhibition of internode elongation, inhibition of petal growth, reduced fertility, promotion of trichome development on sepals, and formation of small dark-green leaves containing higher levels of chlorophyll (Figures 2A to 2F; see Supplemental Figure 3A online). Occasionally, elongated carpels were found in flowers of these lines (see Supplemental Figures 3A and 4 online). Similar phenotypic changes were observed when *TCP15* was expressed under the control of the same promoter (see Supplemental Figure 4 online), suggesting that TCP15 and TCP14 have the potential to be engaged in similar functions.

We tested if TCP14 activity requires functional SPY by analyzing *AS1<sub>pro</sub>>>TCP14 spy-4* plants. All TCP14 overexpression phenotypes (see above) were strongly suppressed by *spy* (Figures 2B to 2F; see Supplemental Figure 3A online). Importantly, the activity of the *AS1* driver remained unchanged in the *spy-4* background, as was evident by the pattern of GFP fluorescence in these lines (see Supplemental Figures 3B and 3C online). Quantitative RT-PCR (qRT-PCR) also confirmed that *TCP14* expression was similar in *AS1<sub>pro</sub>>>TCP14* wild type and *AS1<sub>pro</sub>>>TCP14 spy-4* (Figure 2G). As expected, crossing *AS1<sub>pro</sub>>>TCP14 spy-4* back to the wild type restored the overexpression phenotype (see Supplemental Figure 3A online). These results suggest that at least some TCP14 activities require functional SPY.

### Several TCPs Are O-GlcNAc Modified by SEC

The interaction between SPY and TCP14/15 and its requirement for their activity raised the possibility that these proteins are O-GlcNAc modified by SPY. At present, there is no reliable plant-based system for in vivo analysis of O-GlcNAc modifications, but an *Escherichia coli*-based O-GlcNAc modification assay with SEC (the second *Arabidopsis* OGT) is available (Scott et al., 2006). We first tested if SEC can interact with TCP14/15 and found that they interact in an in vitro pull-down assay (see Supplemental Figure 5 online). We then tested if SEC modifies TCP14 and TCP15. When protein blots containing total proteins from *E. coli* expressing SEC and TCP14 or TCP15 were probed using an assay that labels terminal GlcNAc with <sup>3</sup>H-Gal, both TCP14 and TCP15, but not *E. coli* proteins, were labeled (Figure 3). To determine if TCP14 and TCP15 undergo GlcNAc modification by SEC, they were coexpressed with a mutant form of SEC. This mutant form of SEC has a missense mutation (G676D) that, based on the crystal structure of the *Xanthomonas campestris* OGT (Martinez-Fleites et al., 2010), is predicted to affect the active site. Consistent with SEC-modifying TCP14 and TCP15, the mutation greatly reduced GlcNAc modification of these proteins (Figure 3). Since TCP14 and TCP15 were expressed as GST fusions, SEC could modify GST and not TCP14 or TCP15. However, this is unlikely since another protein (AAR5) fused to GST was not modified by SEC in this assay (see Supplemental Figures 6 and 7 online).

The labeling assay used to detect GlcNAc modifications labels both O- and N-linked GlcNAc modifications. O-linked modifications are removed by high pH ( $\beta$ -elimination), but N-linked modifications



**Figure 2.** Functional Interaction between SPY and TCP14.

**(A)** Four-week-old wild-type (WT) Ler and transactivated transgenic plants overexpressing *TCP14* under the regulation of the *AS1* promoter (*AS1<sub>pro</sub>>>TCP14*). The transgene suppressed leaf expansion and elongation growth.

**(B)** Inflorescences of the wild type, *spy-4*, and *AS1<sub>pro</sub>>>TCP14* in the wild type or *spy-4*.

**(C)** Sepal trichome number of the sixth flower of wild-type Ler (1), *AS1<sub>pro</sub>>>TCP14* (2), *spy-4* (3), and *AS1<sub>pro</sub>>>TCP14* in *spy-4* (4). Values are means of 10 flowers  $\pm$  SE.

**(D)** Flowers (flower no. 6) of wild-type Ler, transgenic transactivated *AS1<sub>pro</sub>>>TCP14*, *spy-4*, and transactivated *AS1<sub>pro</sub>>>TCP14* in *spy-4*.

**(E)** Fifth rosette leaf of wild-type Ler (1), *AS1<sub>pro</sub>>>TCP14* (2), *spy-4* (3), and *AS1<sub>pro</sub>>>TCP14* in *spy-4* (4).

**(F)** Chlorophyll content ( $\mu$ g chlorophyll per mg fresh weight) in the fifth rosette leaf of wild-type Ler (1), *AS1<sub>pro</sub>>>TCP14* (2), *spy-4* (3), and *AS1<sub>pro</sub>>>TCP14* in *spy-4* (4). Values are means of five leaves  $\pm$  SE.

**(G)** qRT-PCR analysis of *TCP14* in seedlings of wild-type Ler (1), *AS1<sub>pro</sub>>>TCP14* (2), *spy-4* (3), and *AS1<sub>pro</sub>>>TCP14* in *spy-4* (4). Values are means of three biological replicates  $\pm$  SE.

Bars = 1 cm in **(A)** and **(E)** and 1 mm in **(B)** and **(D)**.

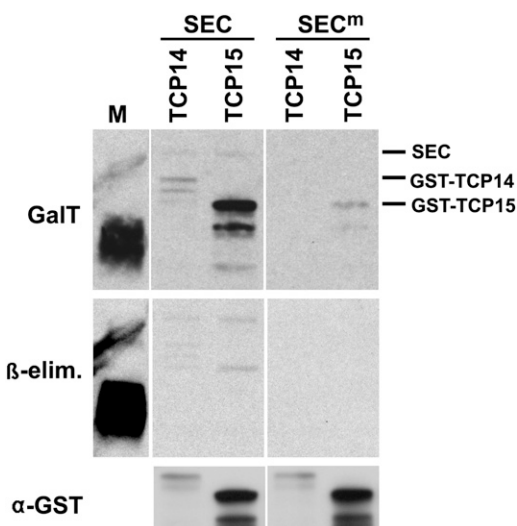
are stable under these conditions. The GlcNAc modifications on TCP14 and TCP15, but not a negative control bearing N-linked modifications, were removed when the blot was subjected to  $\beta$ -elimination, indicating that the GlcNAc modifications on TCP14 and TCP15 are O-linked (Figure 3). These results show that TCP14 and TCP15 have the potential to be O-GlcNAc modified. We tested other TCPs and found that SEC can also modify TCPs 2, 8, 19, 23, and 24 (see Supplemental Figure 7 online). Two other tested proteins were not modified in this assay (see Supplemental Figures 6 and 7 online).

#### The *tcp14 tcp15* Double Mutant Plants Exhibit a Subset of the *spy* Phenotypes

Plants with single and double mutations in *TCP14* and *TCP15* (*tcp14-4* and *tcp15-3*) have been characterized before; in these mutants, a T-DNA lies within *TCP14*'s single exon in the *tcp14-4* line, and no *TCP15* transcripts could be detected in the *tcp15-3*

mutant line (Kieffer et al., 2011). We used these lines (all in the Columbia [Col] background) to examine the relationships between these class I TCPs and SPY. *tcp14* and *tcp15* single mutants have leaf margins slightly smoother than those of wild-type leaves (see Supplemental Figure 8A online). When grown under short-day conditions, the *tcp14 tcp15* double mutant leaf margins were similar to *spy-4* and much smoother than the wild type (Figure 4A). This effect was weaker under long-day conditions (data not shown). As shown before (Kieffer et al., 2011), stem internodes of the double mutant were slightly shorter than the wild type or either of the single mutants.

Mutation of *TCP14* and *TCP15* also affected flower morphology: The wild type and the *tcp14* and *tcp15* single mutants had several trichomes on each sepal (see Supplemental Figure 8B online), but similar to *spy-4*, *tcp14 tcp15* sepals were glabrous (Figure 4B; see Supplemental Figure 8B online). Other typical *spy-4* phenotypes, such as slender growth, early flowering, and



**Figure 3.** TCP14 and TCP15 Are O-GlcNAc Modified by SEC.

GST-tagged TCP14 and TCP15 were coexpressed in *E. coli* with wild-type SEC (SEC) or mutant SEC (SEC<sup>m</sup>). Two identical blots containing total *E. coli* proteins were prepared, and GlcNAc-modified proteins on one blot were labeled with <sup>3</sup>H-Gal (GalT). This blot was then subjected to  $\beta$ -elimination to remove O-linked modifications and exposed to film for twice as long as the original exposure ( $\beta$ -elim.). The second blot was used to monitor TCP14 and TCP15 expression using an anti-GST antibody ( $\alpha$ -GST). Wild-type SEC modified both full-length TCPs and itself to a much greater extent than did SEC<sup>m</sup>.  $\beta$ -Elimination removed most of label from the modified TCPs but not the ovalbumin marker (M), which has N-linked glycans, indicating that the modification is O-linked. The location of SEC, TCP14, and TCP15 on the blot is indicated. The experiment was repeated with similar results.

insensitivity to the GA biosynthesis inhibitor paclobutrazol, were not observed in the double mutant (see Supplemental Figure 9 online). In fact, as reported previously for *tcp14* (Tatematsu et al., 2008), *tcp14 tcp15* seeds were slightly hypersensitive to paclobutrazol. It should be noted, however, that whereas SPY is expressed throughout the plant (Hartweck et al., 2006), TCP14 and TCP15 are expressed primarily in young developing leaves; TCP15 is expressed mainly in leaf margins, whereas TCP14 is expressed more widely in the leaf blade (Kieffer et al., 2011; Li et al., 2011). Both genes are also expressed in young flowers, mainly in sepals, and later in stamens and carpel.

We have found previously that leaf serration and trichome development on sepals are good markers for SPY-promoted CK responses (Greenboim-Wainberg et al., 2005; Maymon et al., 2009). Slender growth, early flowering, and resistance to paclobutrazol, on the other hand, are related to SPY-suppressed GA responses. Thus, it is possible that the interaction of SPY with TCP14/TCP15 has a significant role in CK but not GA responses.

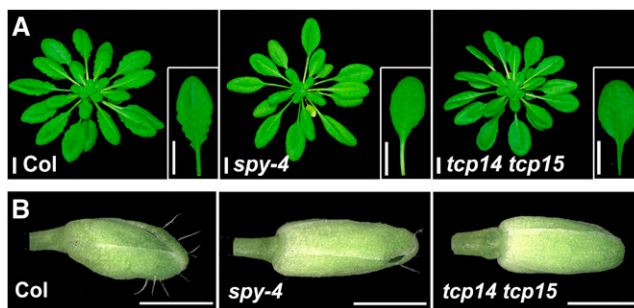
### TCP14 and TCP15 Promote Responses to CK

To test if TCP14 and TCP15 have a role in CK responses, we examined the sensitivity of the *tcp14 tcp15* double mutant to the CK N<sup>6</sup>-benzyladenine (BA). We tested several responses to ex-

ogenous BA, including promotion of leaf serration, inhibition of inflorescence elongation, and trichome development on sepals (Greenboim-Wainberg et al., 2005; Gan et al., 2007; Maymon et al., 2009). Col, *spy-4*, and *tcp14 tcp15* seedlings with two true leaves were sprayed twice a week with 0, 10, or 100  $\mu$ M BA. BA-treated Col plants produced small and highly serrated leaves, but this effect was not apparent in *spy-4* or the *tcp14 tcp15* double mutant (Figure 5A). Furthermore, BA treatment strongly promoted trichome development on wild-type sepals but had a very mild effect on *spy-4* and *tcp14 tcp15* sepals (Figures 5B and 5C). BA treatment also inhibits inflorescence elongation. Whereas *spy* suppresses inhibition of inflorescence elongation by BA (Maymon et al., 2009), the inflorescence of *tcp14 tcp15* plants responds normally to BA, demonstrating that there is incomplete spatial overlap in the functioning of SPY, TCP14, and TCP15.

Some of the phenotypes observed in TCP14- and TCP15-overexpressing plants resemble the effect of CK treatment (e.g., promotion of trichome development on sepals). Furthermore, similar to CK application (Guo and Gan, 2011), TCP14 and TCP15 overexpression promoted shoot branching and delayed whole-plant senescence. Approximately 2 months after germination, when wild-type plants started to die, axillary buds in the transgenic *AS1<sub>pro</sub>>>TCP14* and *AS1<sub>pro</sub>>>TCP15* plants sprouted and produced lateral inflorescences. Three-month-old transgenic *AS1<sub>pro</sub>>>TCP14* and *AS1<sub>pro</sub>>>TCP15* plants were bushy and green (Figure 6A). Although TCP14 and TCP15 overexpression delayed whole-plant senescence, overexpression had no effect on leaf senescence. It should be noted, in this regard, that the *AS1* promoter is not active in mature leaves or in mature inflorescence stems.

To test further if overexpression of TCP14 promotes CK responses, we analyzed the expression of the CK-induced gene *ARR5* in the *AS1<sub>pro</sub>>>TCP14* seedlings. qRT-PCR assays with RNA extracted from whole seedlings revealed an approximately twofold higher expression of *ARR5* in the transgenic



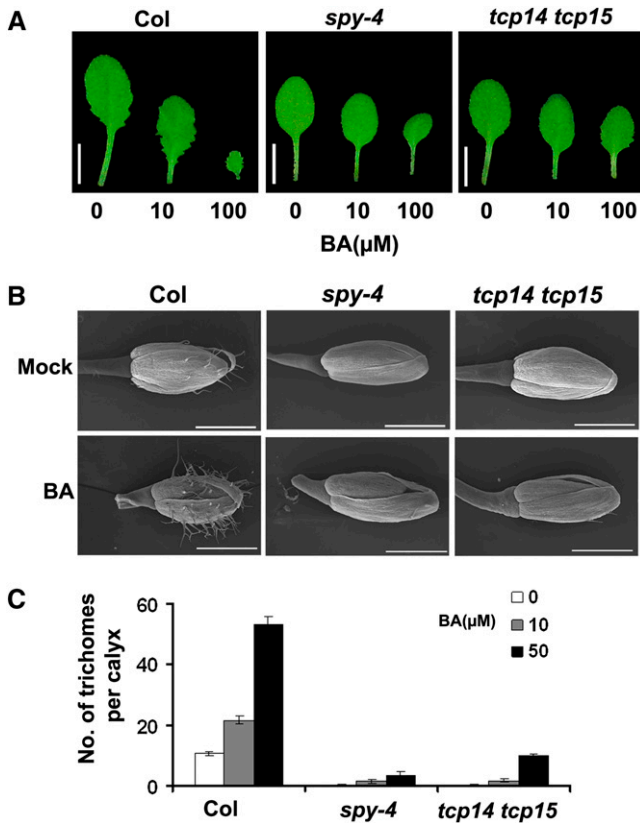
**Figure 4.** Leaf Morphology and Sepal Trichome Development Are Similarly Affected in *tcp14 tcp15* and *spy* Mutants.

(A) Serrated leaves of wild-type Col and smoother leaves of *tcp14 tcp15* and *spy-4*. The seventh leaf of each plant is also shown.

(B) Trichomes on wild-type (Col) sepals and the glabrous sepals of *tcp14 tcp15* and *spy-4* (flower no. 4).

Plants were grown under short-day conditions (8 h of light). Bars = 1 cm in (A) and 1 mm in (B).

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**Figure 5.** Leaves and Flowers of the *tcp14 tcp15* Double Mutant and *spy-4* Are Partially Resistant to CK.

(A) and (B) Wild-type Col, *spy-4*, and *tcp14 tcp15* seedlings were treated with different concentrations of BA for 3 weeks. Representative rosette leaves (fifth leaf; [A]) and flowers (B) of treated and nontreated wild-type (Col), *spy-4*, and *tcp14 tcp15* plants are presented.

(C) Number of trichomes on sepals (first flower) of the wild type (Col), *spy-4*, and *tcp14 tcp15* treated with different BA concentrations. Values are means of 20 flowers  $\pm$  SE.

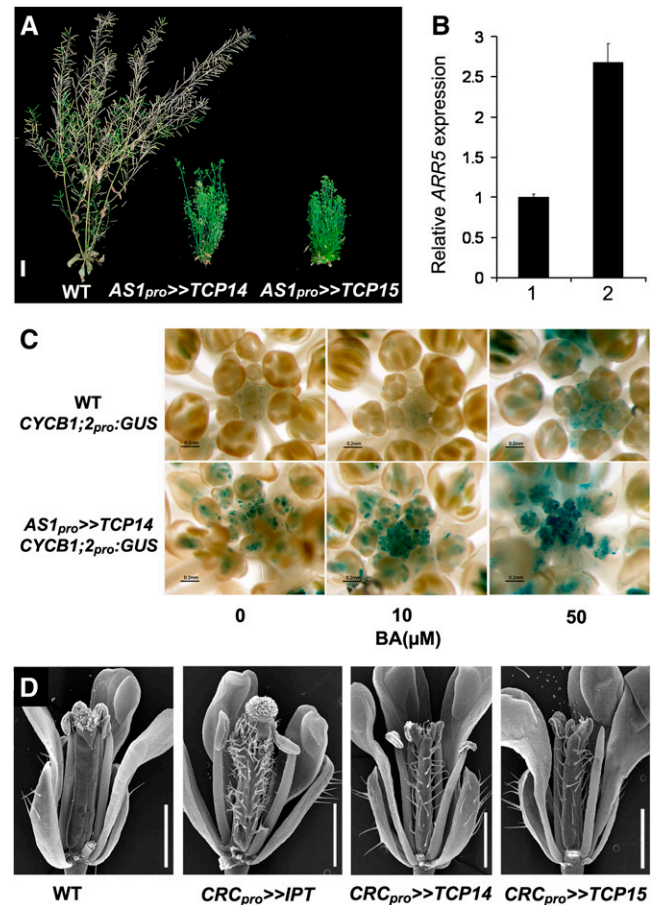
The experiment was performed five times with similar results. Bars = 1 cm in (A) and 1 mm in (B).

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plants compared with the wild type (Figure 6B), suggesting increased CK responses in the transgenic plants.

CK promotes cell proliferation (Riou-Khamlichi et al., 1999), and a previous study has shown that several mitotic factor genes, including *CYCLIN B1;2* (*CYCB1;2*), are downregulated in *tcp14 tcp15* double mutant plants (Kieffer et al., 2011). Since our results suggest that TCP14 promotes CK responses, we tested if TCP14 and CK act in the same pathway to promote mitotic activity by analyzing their effect on *CYCB1;2*. To this end, we used transgenic wild-type and *AS1<sub>pro</sub>>>TCP14* plants expressing the GUS reporter gene under the regulation of the *CYCB1;2* promoter (Efroni et al., 2008). Inflorescences of *CYCB1;2<sub>pro</sub>:GUS* transgenic plants were treated with different BA concentrations and then GUS activity was detected. Transgenic *CYCB1;2<sub>pro</sub>:GUS* inflorescences exhibited very low GUS activity in young flower buds (Figure 6C). GUS activity in *CYCB1;2<sub>pro</sub>:*

*GUS/AS1<sub>pro</sub>>>TCP14* inflorescences was much higher and was maintained longer in developed flowers. CK application promoted *CYCB1;2<sub>pro</sub>:GUS* activity in both wild-type and *AS1<sub>pro</sub>>>TCP14* inflorescences, but the effect of the hormone was much stronger in the *TCP14* transactivated plants. These results support the previous suggestion that TCP14 promotes cell proliferation (Kieffer et al., 2011) and further suggest that it acts in the CK pathway to regulate this process. In addition, the



**Figure 6.** Overexpression of *TCP14* and *TCP15* Caused Typical CK Responses.

(A) Three-month-old wild-type (WT) Ler and transactivated transgenic plants overexpressing *TCP14* or *TCP15*, under the regulation of the *AS1* promoter (*AS1<sub>pro</sub>>>TCP14* and *AS1<sub>pro</sub>>>TCP15*). The transgenes delayed whole-plant senescence and promoted branching.

(B) Overexpression of *TCP14* promoted *ARR5* expression. RNA was extracted from 10-d-old wild-type Ler (1) and *AS1<sub>pro</sub>>>TCP14* (2) seedlings and analyzed by qRT-PCR. Values are means of three biological replicates  $\pm$  SE.

(C) GUS activity in BA-treated inflorescences of wild-type (Ler) and *AS1<sub>pro</sub>>>TCP14* transactivated plants, both expressing the GUS gene under the regulation of *CYCB1;2* promoter.

(D) Flowers (flower no. 2) of control wild-type (Ler) and transactivated plants expressing *IPT*, *TCP14*, or *TCP15* under the regulation of the *CRC* promoter (*CRC<sub>pro</sub>>>IPT*, *CRC<sub>pro</sub>>>TCP14*, and *CRC<sub>pro</sub>>>TCP15*).

Bars = 2 cm in (A), 0.2 mm in (C), and 1 mm in (D).

results suggest that TCP14 enhances the sensitivity of the tissue to CK.

In a previous study, we showed that ectopic expression of the CK biosynthetic gene *ISOPENTENYL TRANSFERASE* (*IPT*) under the regulation of the carpel-specific *CRABS CLAW* (*CRC*) promoter induced trichome development on carpels (Greenboim-Wainberg et al., 2005; Figure 6B). Here, we found that expressing *IPT*, *TCP14*, or *TCP15* under the regulation of the *CRC* promoter in transactivated lines (*CRC<sub>pro</sub>>>IPT*, *CRC<sub>pro</sub>>>TCP14*, and *CRC<sub>pro</sub>>>TCP15*) similarly induced trichome development on carpels (Figure 6D), suggesting increased CK responses in *CRC<sub>pro</sub>>>TCP14* and *CRC<sub>pro</sub>>>TCP15* carpels.

### Reducing CK Levels Suppresses *TCP14* Overexpression Effects

If *TCP14* and *TCP15* promote CK responses, they might, similar to other signaling components in this pathway (Müller and Sheen, 2007), be regulated by CK. We first tested if the expression of these genes is affected by CK by treating 10-d-old wild-type seedlings with 10  $\mu$ M BA. qRT-PCR analyses showed that, while CK treatments promoted *ARR5* expression, it had no effect on *TCP14* and *TCP15* mRNA levels (see Supplemental Figure 10 online). We then tested if CK affects *TCP14* activity. To this end, we transactivated the *Arabidopsis* CK-degrading *CK OXIDASE/DEHYDROGENASE3* (*CKX3*) gene (Shani et al., 2010) with the *AS1<sub>pro</sub>* driver line. The *CKX3* genes have been used before for the generation of CK-deficient plants (Werner et al., 2003). *AS1<sub>pro</sub>>>CKX3* plants were relatively normal but exhibited late flowering (Figures 7A and 7B). This effect of *CKX3* overexpression was shown before (Werner et al., 2003). We then crossed *AS1<sub>pro</sub>>>CKX3* to *AS1<sub>pro</sub>>>TCP14* to generate *AS1<sub>pro</sub>>>TCP14/CKX3* plants. Progenies containing all three transgenes were identified by PCR (see Supplemental Figure 11D online) and their phenotype was examined. While *AS1<sub>pro</sub>>>TCP14/CKX3* plants showed the typical late-flowering phenotype of *AS1<sub>pro</sub>>>CKX3* (Figures 7A and 7B), two *AS1<sub>pro</sub>>>TCP14* phenotypes, inhibition of petal development and promotion of trichome development on sepals, were strongly suppressed (Figure 7C; see Supplemental Figures 11B and 12 online). Other effects of *AS1<sub>pro</sub>>>TCP14* were only partially suppressed by *CKX3* activity; leaves and siliques of *AS1<sub>pro</sub>>>TCP14/CKX3* plants were larger than those of *AS1<sub>pro</sub>>>TCP14* plants but smaller and darker green than those of wild-type and *AS1<sub>pro</sub>>>CKX3* plants (see Supplemental Figures 11A to 11C online). qRT-PCR analysis showed that the *TCP14* expression level was similar in *AS1<sub>pro</sub>>>TCP14* and in *AS1<sub>pro</sub>>>TCP14/CKX3* plants (Figure 7D). To confirm the presence of active *TCP14* in these plants, *AS1<sub>pro</sub>>>TCP14/CKX3* plants (heterozygous for both transgenes) were self-pollinated. Four phenotypic classes, the wild type, *CKX3*<sup>-</sup>, *CKX3/TCP14*<sup>-</sup>, and *TCP14*-like, were recovered, confirming the presence of active *TCP14* in *AS1<sub>pro</sub>>>TCP14/CKX3* parental plants.

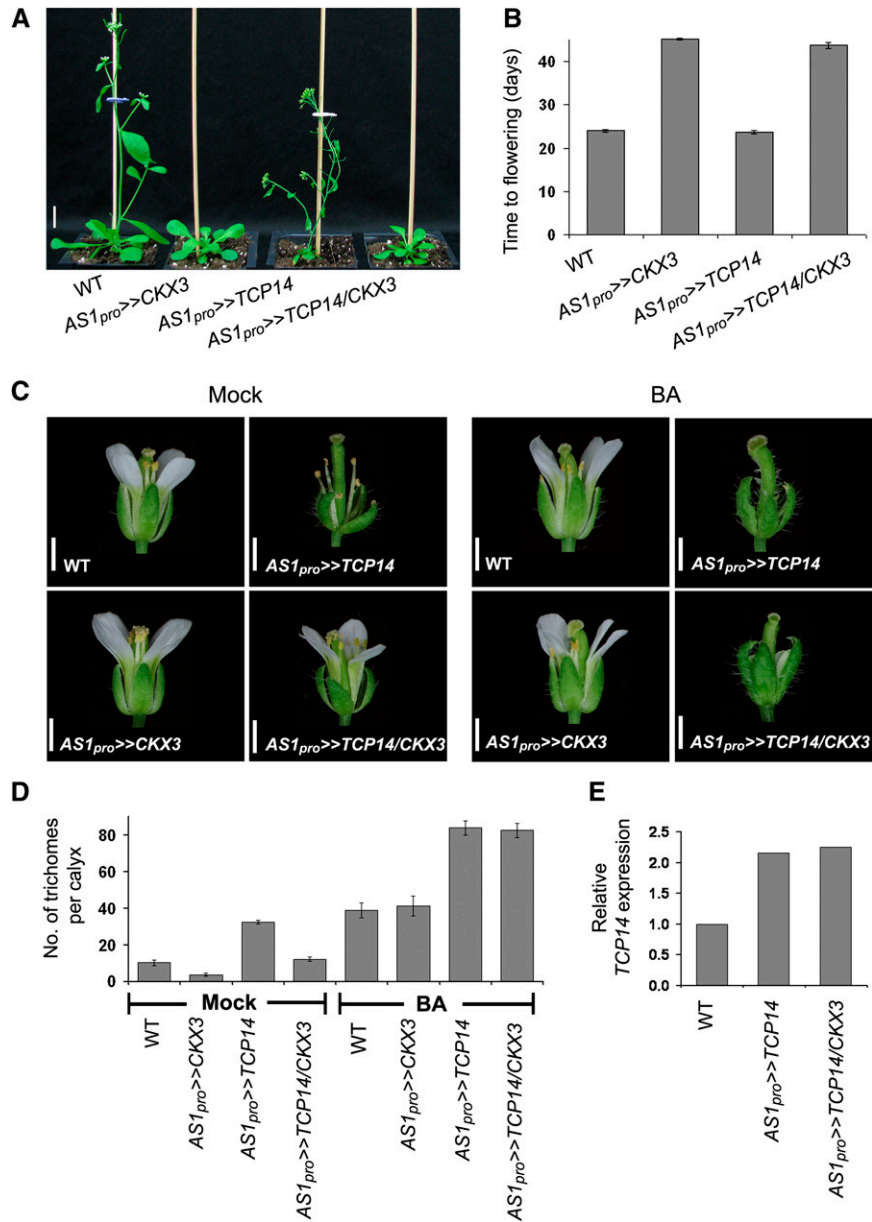
To examine if the *TCP14* overexpression phenotypes in *AS1<sub>pro</sub>>>TCP14/CKX3* plants were suppressed due to the low CK levels, we applied 50  $\mu$ M BA directly to the young inflorescences of the different lines (the wild type, *AS1<sub>pro</sub>>>TCP14*, *AS1<sub>pro</sub>>>CKX3*, and *AS1<sub>pro</sub>>>TCP14/CKX3*). CK application to

*AS1<sub>pro</sub>>>TCP14/CKX3* restored the *TCP14* overexpression phenotype, including the development of trichomes on sepals and inhibition of petal growth (Figure 7C; see Supplemental Figure 12 online). CK application to *AS1<sub>pro</sub>>>TCP14* plants strongly promoted the development of numerous trichomes on sepals (see Supplemental Figure 12 online), in agreement with *TCP14*-overexpressing plants being hypersensitive to CK. These results suggest that *CKX3* suppresses the effects of *TCP14* overexpression by reducing the CK level. Thus, while the most logical explanation for these results is that CK promotes *TCP14* activity, we cannot exclude the possibility that *TCP14* upregulates CK levels.

### DISCUSSION

The plant OGT SPY can affect developmental processes in *Arabidopsis* by suppressing GA signaling and/or promoting CK responses (Jacobsen and Olszewski, 1993; Greenboim-Wainberg et al., 2005). The mechanism by which SPY influences plant development and hormone responses is still unknown. Here, we report that SPY physically interacts with two class I TCPs: *TCP14* and its closest homolog *TCP15*. We also show that functional SPY is required for *TCP14* activity and provide evidence that *TCP14* and *TCP15* act together with SPY to promote CK responses. Whereas there is no direct evidence, it is possible that O-GlcNAc modification by SPY activates *TCP14* and *TCP15* to facilitate specific CK responses in leaves and flowers. The observation that *E. coli*-expressed SEC modifies *TCP14* and *TCP15* demonstrates that these proteins have the potential to be O-GlcNAc modified in planta. It should be noted that *sec* mutants do not exhibit phenotypes consistent with action in CK signaling or in *TCP14/15* activity. Thus, it is possible that, whereas SEC can interact in vitro with *TCP14* and *TCP15* and modify them in *E. coli*, differences in expression pattern or cellular localization prevent it from modifying them in planta.

Several lines of evidence suggest that *TCP14* and *TCP15*, following activation by SPY, promote specific CK responses: Loss of *TCP14* and *TCP15* function, similar to loss of SPY, suppressed marginal leaf serration and trichome development on sepals and attenuated the effect of exogenous CK on these processes. Furthermore, the phenotype of plants overexpressing *TCP14* (and *TCP15*) was similar to that of plants treated with high doses of CK; both *TCP14* overexpression and CK promoted branching and trichome formation on sepals and inhibited inflorescence elongation, leaf growth, and petal elongation (Greenboim-Wainberg et al., 2005; Maymon et al., 2009; Figures 2 and 5 to 7). These effects of *TCP14* overexpression were strongly suppressed by the loss of SPY activity. Overexpression of *TCP14* also promoted the expression of the CK response gene *ARR5*. Moreover, expressing *TCP14* or *TCP15* in the carpel had a similar effect to the expression of the CK-biosynthetic gene *IPT*; all promoted trichome development on the normally glabrous carpel. *TCP14* overexpression also increased the activity of the promoter of the mitotic factor *CYCB1;2* and enhanced the responsiveness of this promoter to CK. This result suggests that *TCP14* overexpression not only promotes CK responses but also increases the sensitivity of the



**Figure 7.** Reducing CK Levels Suppresses TCP14 Overexpression Effects.

**(A)** Four-week-old wild-type (WT) Ler, transactivated  $AS1_{pro}>>CKX3$ , transactivated  $AS1_{pro}>>TCP14$ , and transactivated  $AS1_{pro}>>TCP14/CKX3$  (expressing both *CKX3* and *TCP14*) plants. Bar = 1 cm.

**(B)** Time to flowering of the wild type and the different transgenic lines. The number of days from germination to production of a 2-cm-long inflorescence was determined. Values are means of 10 plants  $\pm$  SE.

**(C)** Flowers (flower no. 4) of wild-type Ler,  $AS1_{pro}>>TCP14$ ,  $AS1_{pro}>>CKX3$ , and  $AS1_{pro}>>TCP14/CKX3$  nontreated (left) and treated (right) with 50  $\mu$ M BA. Bars = 1 mm.

**(D)** Sepal trichome number of the third flower of wild-type Ler,  $AS1_{pro}>>CKX3$ ,  $AS1_{pro}>>TCP14$ , and  $AS1_{pro}>>TCP14/CKX3$  nontreated or treated with 50  $\mu$ M BA. Values are means of 10 flowers  $\pm$  SE.

**(E)** qRT-PCR analysis of *TCP14* in inflorescences of wild-type Ler,  $AS1_{pro}>>TCP14$ , and  $AS1_{pro}>>TCP14/CKX3$  plants. The analysis was repeated three times with different plants and crosses, and similar results were obtained.

[See online article for color version of this figure.]



tissue to the hormone. The observations that *TCP14* overexpression also enhanced the effects of CK on sepal trichome development further support this suggestion.

Whereas CK-promoted marginal leaf serration was suppressed in *tcp14 tcp15*, it was not affected by *TCP14* overexpression. This discrepancy is probably caused by the different genetic backgrounds used in the different experiments. Whereas *tcp14 tcp15* is in the Col background, *TCP14* overexpression is in Landsberg *erecta* (*Ler*). Col leaves are slightly serrated, and CK strongly increases serration. *Ler* leaves, on the other hand, are smooth even after CK treatment.

Taken together, the results suggest that *TCP14* and *TCP15*, similar to *SPY*, promote CK responses. However, while the loss of *SPY* affects CK responses of seedling and mature plants organs (Greenboim-Wainberg et al., 2005), the loss of *TCP14/TCP15* suppressed CK activity only in leaves and sepals. Thus, *TCP14* and *TCP15* may mediate only a subset of *SPY*-regulated CK responses. This is expected, as *SPY* and *TCP14/TCP15* expression have limited spatial overlap; *SPY* is expressed throughout the plant (Hartweck et al., 2006), while *TCP14* and *TCP15* are expressed mainly in emerging lateral roots, young leaves, and flowers (Kieffer et al., 2011; Li et al., 2011). It is possible, however, that additional TCPs are activated by *SPY* in other tissues/developmental stages to promote other CK responses. The fact that *TCP8*, *TCP22*, and *TCP23*, which are closely related to *TCP14* and *TCP15*, could be O-GlcNAc modified by *SEC* supports this possibility.

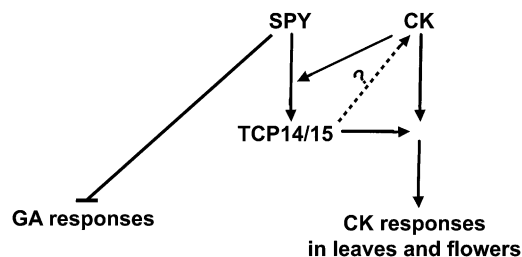
Whereas very little is known about the developmental role of class I TCPs, it was proposed that they promote cell proliferation (Li et al., 2005; Martín-Trillo and Cubas, 2010; Kieffer et al., 2011; Li et al., 2011). Cell proliferation is also promoted by CK, and both class I TCPs (i.e., *TCP20*, *TCP14*, and *TCP15*) and CK enhance the expression of cyclin genes to regulate this process (Riou-Khamlichi et al., 1999; Li et al., 2005; Kieffer et al., 2011; Li et al., 2011; Figure 6). We show that *TCP14* overexpression promotes the expression of the mitotic factor *CYCB1;2* and enhances the induction of this gene by CK. This implies that *TCP14* and *TCP15* mediate the effect of CK on *CYC* genes to promote cell proliferation. Recently, it was shown that CK promotes cell proliferation at the leaf margin, leading to increased leaf serration and complexity (Shani et al., 2010). Both leaf margin serration and the promotion of it by CK are attenuated in *spy* and *tcp14 tcp15* plants. Therefore, it is possible that *TCP14* and *TCP15*, following activation by *SPY*, act in the CK pathway to promote cell proliferation at the leaf margin. This suggestion is supported by the expression pattern of these TCPs: *TCP14* is expressed in leaf blades, and *TCP15* is expressed mainly in leaf margins (Kieffer et al., 2011).

Our results show that reducing the endogenous levels of CK by overexpressing the CK-degrading enzyme *CKX3* suppressed *TCP14*-induced trichome development on sepals and inhibition of petal development. These effects were restored when the plants (*AS1>>TCP14/CKX3*) were treated with exogenous CK. Several different models that are not mutually exclusive can explain these results; *TCP14* could increase the CK level either by promoting its biosynthesis or inhibiting its degradation or CK signaling could promote *TCP14* protein activity (Figure 8). Alternatively, *TCP14* could perform a step in the CK signaling pathway

that is rate limiting when CK levels are normal but not when CK levels are reduced by overexpression of *CKX*. The resistance of *tcp14 tcp15* mutant to exogenously applied CK suggests that *TCP14* and *TCP15* are involved in CK signaling and not biosynthesis. Thus, we favor the possibility that CK signaling activates *TCP14* and *TCP15*.

Overexpression of *TCP14* and *TCP15* caused some phenotypic changes found in plants with suppressed GA responses (e.g., dwarfism and dark-green small leaves). These typical GA-regulated responses were suppressed by the loss of *SPY*, suggesting that *TCP14* and *TCP15* also mediate the suppression of GA responses by *SPY*. However, this is not supported by the phenotype of the double mutant *tcp14 tcp15*: Whereas a mutation in *SPY* affects typical GA responses, such as seed germination, cell elongation, and flowering time, the *tcp14 tcp15* double mutant was not affected in these or other GA responses. Moreover, whereas *spy* is resistant to the GA biosynthesis inhibitor paclobutrazol, *tcp14* and *tcp14 tcp15* mutants are hypersensitive to the chemical (Tatematsu et al., 2008; see Supplemental Figure 9 online). Since different TCPs bind similar *cis*-regulatory elements (Martín-Trillo and Cubas, 2010), it is possible that some of the *TCP14/15* overexpression phenotypes are caused by nonspecific activation/suppression of target genes, which are normally regulated by other TCPs. By that scenario, other TCPs may have a role in *SPY*'s suppression of GA responses, and, notably, *SEC* can modify several class I and class II TCPs.

A previous study has shown that *SPY* affects the activity of the circadian clock; mutations in *SPY* lengthen the free-running period of cotyledon movements, and overexpression of the gene shortens it (Tseng et al., 2004). It was proposed that *SPY* acts together with *GI* in the circadian clock pathway. Recently, several studies have shown interactions of clock elements such as *TOC1* and *PRR5* with different TCPs, including *TCP15* and *TCP21/CHE* (Pruneda-Paz et al., 2009; Giraud et al., 2010). Since we show here that *TCP15* interacts with *SPY*, it is possible that at least part of *SPY*'s effect on clock activity is mediated by *TCP15*.



**Figure 8.** A Model for the Interaction between *SPY* and *TCP14/TCP15* and Its Effect on CK Responses.

Our results demonstrate that *SPY* interacts with *TCP14* and *TCP15* to facilitate CK responses in leaves and flowers. They also suggest that CK promotes *TCP14* protein activity via *SPY*-dependent or -independent pathways. As the effects of *TCP14* are CK dependent, it is possible that activated *TCP14/15* can also regulate CK levels (dotted arrow with question mark). By contrast, *SPY* suppresses GA responses via a *TCP14/TCP15*-independent pathway.

In conclusion, the results of this study suggest that at least part of SPY's activity in the regulation of plant development is mediated by class I TCP transcription factors. We propose that SPY interacts with TCP14 and TCP15 to promote CK effects in flowers and leaves (Figure 8). Such effects may be mediated by changes in CK signaling effectors or, alternatively, via increased CK levels in leaves and flowers, either by promoting its biosynthesis or inhibiting its degradation. As SPY also suppresses GA responses via a TCP14/TCP15-independent pathway, its pivotal role as a hub in hormone signaling integration is becoming evident.

## METHODS

### Plant Material, Growth Conditions, and Genetics

The *Arabidopsis thaliana* *spy-4* mutant plants used in this study are in the Col-0 and in *Ler* backgrounds (Maymon et al., 2009). Single *tcp14-4* and *tcp15-3* and double *tcp14 tcp15* mutants are in the Col-0 background (Kieffer et al., 2011). *TCP14*, *TCP15*, and *CKX3* overexpression phenotypes and *CYCB1;2<sub>pro</sub>:GUS* are in *Ler* background. *Arabidopsis* seeds were sterilized, cold treated, and germinated on sterile Murashige and Skoog medium or in pots. Plants were grown in a growth room under controlled temperature (22°C) and long (16 h light/8 h dark) or short (8 h light/16 h dark) days.

### Hormone Treatments

#### BA Treatment

*Arabidopsis* seedlings with two true leaves were sprayed with different concentrations of BA (Sigma-Aldrich) twice a week for ~3 weeks. In some cases, BA was applied directly to the inflorescence of mature plants.

#### Paclubutrazol Treatment

*Arabidopsis* seeds were germinated on plates containing Murashige and Skoog medium (Duchefa Biochemie), 0.8% (w/v) agar, and 1.5% (w/v) Suc with or without 2 mg L<sup>-1</sup> paclobutrazol.

### Scanning Electron Microscopy

Samples for scanning electron microscopy were fixed in 5% (w/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, transferred to ethanol (25% up to 100%), critical point dried with liquid carbon dioxide in a CPD 750 (Bio-Rad), sputter-coated with gold, and photographed with a Jeol scanning electron microscope (JSM-5410 LV).

### Yeast Two-Hybrid Assays

Clones containing the full *TCP14* and *TCP15* coding regions were obtained from the ABRC. The *TCP14* and *TCP15* coding regions were PCR amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The primers introduced *NdeI* and *XhoI* sites flanking the coding regions (primer sequences are detailed in Supplemental Table 1 online). After adding 3' A-overhangs, the PCR products were cloned into pCR2.1-TOPO (Invitrogen). The coding regions were subcloned into the prey vector pGADT7 (Clontech) as *NdeI* to *XhoI* fragments. The bait plasmid expressing the SPY TPR region was described previously (Tseng et al., 2001). Bait and prey plasmids were transformed into the *Saccharomyces cerevisiae* strain HF7c, and the two hybrid screen was performed as described by Bai and Elledge (1997).

### In Vitro Pull-Down Assays

*TCP14* and *TCP15* coding regions were cloned into pDEST15 (Invitrogen), and *SEC* and *SPY* coding regions were cloned into pRARE-MBP-DEST. The pRARE-MBP-DEST Gateway cloning vector expresses MBP-tagged proteins under the control of the isopropyl-β-D-thiogalactoside (IPTG)-inducible *p<sub>tac</sub>* promoter and also expresses several tRNAs that are rare in *Escherichia coli*. The vector was constructed by modifying the *malE* expression cassette of pMAL-c2 (New England Biolabs) for Gateway cloning. The region containing the *lacI<sup>q</sup>* gene and modified *malE* expression cassette was then subcloned into the pRARE plasmid (EMD Chemicals). The detailed sequences of primers used for cloning are presented in Supplemental Table 1 online. MBP and GST were expressed from pMALC1 (New England Biolabs) and pGEX (GE Healthcare), respectively. Plasmids for encoding MBP-SEC and MBP-SPY were expressed in the BL21-AI strain of *E. coli* (Invitrogen), and the other proteins were expressed in the Rosetta 2 strain (EMD Chemicals). For protein expression, cells were grown in Luria-Bertani medium containing 0.1% Glc and either 25 μg/mL chloramphenicol for BL21-AI cells or 25 μg/mL chloramphenicol and 75 μg/mL ampicillin for Rosetta 2 cells at 25°C. Protein expression was induced by the addition of IPTG to 1 mM to Rosetta 2 cultures or both IPTG and Ara to 0.2% to BL21-AI cultures when the OD<sub>600</sub> was between 0.4 and 0.6. The cultures were grown for an additional 2 h, the cells were collected by centrifugation, and the cell pellet was stored at -20°C. Cell lysates were prepared as described previously (Tseng et al., 2004). We quantitated the prey (MBP, MBP-SEC, and MBP-SPY) and bait (GST, PST-TCP14, and GST-TCP15) proteins in the respective cell lysates by probing an immunoblot containing a dilution series for each lysate with anti-MBP or anti-GST, respectively. By comparing the signals from the different dilution series, it was possible to determine the relative amount of prey or bait in each lysate. Lysate mixtures for the different combinations of MBP-tagged and GST-tagged proteins were prepared. The MBP-tagged protein was pulled down, and immunoblots containing the pulled-down proteins were prepared as described previously (Tseng et al., 2004). GST-tagged proteins were detected using anti-GST horseradish peroxidase (HRP) conjugate (GE Healthcare Biosciences). The blot was then stripped and probed with anti-MBP HRP conjugate (New England Biolabs).

### Generation of Transgenic Plants and the Transactivation System

The coding regions of both *TCP14* and *TCP15* were cloned in *XhoI* and *HindIII* sites under the control of an op array into pBJ36 (Moore et al., 1998), subcloned into the pMLBART binary vector (Goldshmidt et al., 2008), and transformed into *Ler* plants by floral dipping. At least 10 different T1 lines were examined, and representative lines with a single insertion were selected for further analysis. *AS1<sub>pro</sub>:LhG4* (Sarojam et al., 2010), *FIL<sub>pro</sub>:LhG4*, *CRC<sub>pro</sub>:LhG4*, *op:GFP* (Goldshmidt et al., 2008), *op:CKX3* (Shani et al., 2010), *CYCB1;2<sub>pro</sub>:GUS* (Efroni et al., 2008), and *CRC<sub>pro</sub>>>IPT* (Greenboim-Wainberg et al., 2005) plants were previously described. *CUC2<sub>pro</sub>:LhG4* was generated with PCR-based cloning of 3480 bp 5' to the gene's ATG, and its expression largely matched the published *CUC2* expression (primer sequences are detailed in Supplemental Table 1 online). Lines were crossed to generate the genotypes described in the article.

### qRT-PCR and PCR Analyses

qRT-PCR analysis was performed using the Absolute Blue qPCR SYBR Green ROX Mix (AB-4162/B) kit (Thermo Fisher Scientific). Reactions were performed using a Rotor-Gene 6000 cycler (Corbett Research). A standard curve was obtained for each gene using dilutions of a cDNA sample. Each gene was quantified using Corbett Research Rotor-Gene software. At least three independent technical repeats were performed for each cDNA sample. Relative expression of each sample was calculated by dividing the

expression level of the analyzed gene by that of *TUBULIN*. Gene-to-tubulin ratios were then averaged and presented as a proportion of the control treatment, set to a value of 1. All sequences of primers used for PCR and qRT-PCR analyses are presented in Supplemental Table 1 online.

### O-GlcNAc Modification Assay

The *SEC*, *TCP14*, and *TCP15* coding regions were amplified (primer sequences are detailed in Supplemental Table 1 online) using Pfx50 DNA polymerase (Invitrogen). The PCR products were Gateway cloned into pDONR221 (Invitrogen). The *SEC* coding region was transferred by the Gateway reaction from pDONR221 into the *E. coli* expression vector pCOLA-2-DEST (EMD Chemicals), and the *TCP14* and *TCP15* coding regions were transferred into the *E. coli* expression vector pDEST15 (Invitrogen). Rosetta 2 (DE3) *E. coli* cells were cotransformed with the pCOLA-2-DEST plasmid encoding *SEC* and the pDEST15 plasmid encoding one of the TCPs. The plasmids were selected using chloramphenicol (25  $\mu$ g/mL) and ampicillin (75  $\mu$ g/mL). Five-milliliter cultures were grown overnight with antibiotic selection at 37°C in Luria-Bertani medium and then 20-mL cultures were inoculated with  $\sim$ 1 mL of the saturated culture and grown at 22°C. At an OD<sub>600</sub> of 0.4, *SEC* and TCP expression were induced by the addition of IPTG to a final concentration of 1.0 mM. Two hours after the induction of protein expression, cells from 1.5 mL of culture were harvested by centrifugation. The bacterial pellets were resuspended in 80  $\mu$ L of SDS sample buffer, boiled for 5 min, and, if not used immediately, stored at  $-20^{\circ}\text{C}$ . Fifteen microliters of each samples were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore). Terminal GlcNAc on membrane-bound proteins was detected by labeling with [<sup>3</sup>H] Gal, and the label was detected by fluorography (Scott et al., 2006). To determine if the modification was O- or N-linked, the blot was subjected to  $\beta$ -elimination (Duk et al., 1997). The blot was washed three times for 30 min in 95% ethanol to remove fluorography agent. It was then equilibrated in double distilled water for 5 min followed by equilibration in 55 mM NaOH for 5 min. The blot was then incubated in 55 mM NaOH for 16 h at 40°C and then washed four times in 10 mM HEPES, pH 7.9, for 5 min and air dried, and the residual radioactivity was detected by fluorography. Protein expression was assessed by probing duplicate blots with anti-GST HRP conjugate (GE Healthcare), which detects the TCP fusion proteins, or HisProbe-HRP (Thermo Scientific), which detects His-tagged *SEC*.

### Detection of GUS Activity

Histochemical detection of GUS activity was performed using 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide as described before (Donnelly et al., 1999).

### Chlorophyll Measurements

Chlorophyll was extracted and measured spectrophotometrically from fresh leaves (fifth leaf) and normalized to fresh weight (Arnon, 1949).

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ARR5*, At3g48100; *CKX3*, At5g56970; *CUC2*, At5g53950; *CYCB1;2*, At5g06150; *SEC*, At3g04240; *SPY*, At3g11540; *TCP14*, At3g47620; and *TCP15*, At3g69690.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** *SPY* Does Not Interact with Free GST.

**Supplemental Figure 2.** Overexpression of *TCP14* under the Regulation of *CUC2* or *FIL* Promoters Only Slightly Affected Plant Development.

**Supplemental Figure 3.** *TCP14* Activity Requires Functional *SPY*.

**Supplemental Figure 4.** Overexpression of *TCP14* and *TCP15* Suppresses Petal Development.

**Supplemental Figure 5.** *SEC* Interacts with *TCP14* and *TCP15*.

**Supplemental Figure 6.** *SEC* Does Not Modify *ARR5* or *GST*.

**Supplemental Figure 7.** *SEC* Modifies *TCP 2, 8, 19, 23, and 24*.

**Supplemental Figure 8.** The Double Mutant *tcp14 tcp15* and *spy* Share Similar Phenotypes.

**Supplemental Figure 9.** The loss of *TCP14* and *TCP15* Has No Effect on GA Responses.

**Supplemental Figure 10.** Expression of *ARR5*, but Not *TCP14* or *TCP15*, Is Promoted by CK.

**Supplemental Figure 11.** Reducing CK Levels Suppresses *TCP14* Overexpression Effects.

**Supplemental Figure 12.** Overexpressing *CKX3* Suppresses *TCP14* Activity and This Is Reversed by Exogenous CK.

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### AUTHOR CONTRIBUTIONS

D.W., Y.E., and N.O. designed the research. E.S., I.E., M.G., T.-S.T., and M.K. performed research. E.S., D.W., Y.E., K.S., and N.O. wrote the article.

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